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Lipid alterations in Farber Disease

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Resumo

A doença de Farber é uma esfingolipidose fatal, causada pela deficiente atividade da ceramidase ácida.

Esta hidrolase é responsável pela degradação da ceramida em esfingosina e ácidos gordos livres no interior dos lisossomas. Embora os avanços científicos sejam consideráveis, no conhecimento das bases genética e bioquímica da deficiência em ceramidase ácida, pouco é o que se sabe acerca da patogenesis molecular da doença.

Um evento patogénico comum em diversas esfingolipidoses pode residir no bloqueio de transporte intracelular do lisossoma ou para o lisossoma, em pontos celulares relacionados e com consequente atraso endossomal/lisossomal.

O presente estudo investiga alterações lipídicas secundarias em fibroblastos de doentes de Farber bem como a existência e localização de domínios ordenados compostos por C16- ou C18-ceramida, e colesterol nas diferentes membranas biológicas em diferentes linhas celulares, dando enfâse à doença de Farber.

A marcação intensa pela coloração de Filipina, é indicativa de altos níveis intracelulares de colesterol não esterificado, e foi observado depois da sobrecarga com LDL, em doentes com deficiência em ceramidase ácida.

Foi obtida uma observação semelhante com o gangliosido GM2. Através da microscopia de fluorescência, o BODIPY-LacCer é endocitado a partir da membrana plasmática até aos endosomas/lysosomas, em contraste com o controlo negativo onde o análogo lipídico foi transportado até ao complexo de Golgi.

Relativamente ao BODIPY-C5-Cer, um aumentos dos níveis no complexo de Golgi foi observado em doentes de Farber. Quando os fibroblastos de doentes de Farber foram marcados com o anticorpo anti C16-Cereramida/Colesterol, foi observado um aumento dos níveis dos domínios Ceramida/Colesterol em doentes do tipo 4 e 7, as formas mais severas da doença. Depois da sobrexpressão da ceramidase ácida verificou-se a correção do tráfego observado em fibroblastos de doentes de Farber, deficientes em ceramidase ácida, e observou-se que ao reduzir os níveis de ceramida ou colesterol os domínios ceramida/colesterol diminuíram.

Estes dados fornecem uma importante evidência do nível de ceramida lisossomal para a homeostasia lipídica e que, quando alterados, pode levar a um nível de desequilíbrio de esfingolípidos e colesterol e, subsequentemente, a alterações nas vias de sinalização celular específicos, com impacto no tráfico intracelular de lípidos e domínios lipídicos.

Palavras-Chave

Doença de Farber, Ceramida, domínios lipídicos, doença de armazenamento.

Abstract

Farber disease (FD) is a fatal sphingolipidosis caused by the deficient activity of acid ceramidase (ACDase). This hydrolase is responsible for the degradation of ceramide (Cer) into sphingosine and free fatty acid in lysosomes. Although some advances have been made in the knowledge of the genetic and biochemical bases of ACDase-deficiency, little is known about the disease molecular pathogenesis. One pathogenic event common to several sphingolipidoses could reside in blocking of intracellular transport to or from lysosomes at several related cellular points with subsequent endosomal/lysosomal jam.

In this study we investigated secondary lipid accumulations in FD fibroblasts patients as well as the existence and location of ordered domains composed of C16- or C18-ceramide and cholesterol (Chol) in different biological membranes in different types of cells, giving emphasis to FD.

Increased filipin staining, indicative of high levels of intracellular unesterified Chol, was observed in ACDase-deficient cells after LDL overload. A similar observation was made for the ganglioside GM2. By fluorescence microscopy BODIPY-LacCer was found to be endocytosed from the plasma membrane (PM) to endosomes/lysosomes, in contrast to normal control cells where the lipid analog was transported to the Golgi complex. For BODIPY-C5-Cer, an increased level at the Golgi complex was seen in FD cells. Labeling of fibroblasts from patients with FD with an anti-C16-Cer/Chol antibody revealed an elevation in the Cer/Chol domains in type 4 and 7 FD, the most severe form of the disease. Overexpression of ACDase corrected lipid traffic imbalances observed in ACDase-deficient fibroblasts, and reducing Cer or Chol levels could modulate the Cer/Chol domains elevation.

These data provide evidence for the importance of a steady-state level of lysosomal Cer for lipid homeostasis that, when disrupted, may lead to an unbalanced level of sphingolipids and Chol and subsequently, to alterations in specific cell signaling pathways with impact in intracellular lipid trafficking and lipid domains.

Keywords

Farber disease, ceramide, lipid domains, storage disease.

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List of Abbreviations

ACDase- Acid ceramidase
AL- Autophagolysosome
AP- Autophagosome
ASMase- Acid sphingomyelinase
ATPases- Adenosine triphosphatases
AV- Autophagic vacuoles
BCA - Bicinchoninic acid
BMT- Bone marrow transplantation
BODIPY-C5-Cer-N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)
BODIPY-C5-SM - N-[5-(5,7-dimethyl boron dipyrromethene difluoride)-1-pentanoyl]-D-*erythro*-sphingomyelin
BODIPY-LacCer - BODIPY-lactosylceramide
BSA - Bovine serum albumin
CD - 2-hydroxypropyl-β-cyclodextrin
Cer – Ceramide
Chol – Cholesterol
CLN-3 - Neuronal ceroid lipofuscinoses type 3
CNS - Central nervous system
DAPI - 4'6-diamidino-2-phenylindole
DMEM - Dulbecco's Modified Eagle medium
ESCRT - Endosomal sorting complex required for transport
EDTA - Ethylenediamine tetraacetic acid
EE- Early endosomes
ER-Endoplasmic reticulum
FBS - Fetal bovine serum
FD – Farber disease
GAG – Glycosaminoglycans
GM1 - GM1-gangliosidosis
GSL- Glycosphingolipid
HDF – Human skin fibroblasts
HMU-PC - 6-hexadecanoylamino-4-methylumbelliferylphosphorylcholine
HPTLC - Lipid Extractions and High performance thin layer chromatography
ICD - I-cell disease
LAMP-1 - Lysosomal associated membrane protein type 1
LAMP-2 - Lysosomal associated membrane protein type 2

LDS- Lysosomal storage disorder

LE- Late endosome

LIMP-2 - Lysosomal integral membrane protein type 2

LPDS - Lipoprotein-deficient Serum

MLB- Multilamellar bodies

MLD – Mucopolysaccharidoses

M6P - Mannose 6- phosphate

MPRs - Mannose 6-phosphate receptors

MVB - Multi-vesicular body

NCL - Neuronal ceroid lipofuscinosis

NPB - Niemann-Pick type B

NPD - Niemann–Pick Disease

NPC1- Niemann-Pick disease type C1

OSBP - Oxysterol binding protein

PAS- Pre-autophagic structure

PM – Plasma membrane

pSAP - Sphingolipid activator protein precursor

pSAP-D - Sphingolipid activator protein precursor D

RER- Rough endoplasmic reticulum

SAP- Sphingolipid activator protein

SL- Sphingolipid

SM – Sphingomyelin

S1P- Sphingosine-1-phosphate

SPT- Serine palmitoyl transferase

TEM - Transmission electron microscopy

TGN-Trans-Golgi network

TLC - Thin-layer chromatography

TNF- Tumor necrosis factor

VDAC1 - Anti-voltage-dependent anion-selective channel protein 1

WGA - Wheat germ agglutinin

WT- Wild Type

Introduction

Introduction

1. Lysosomes

Lysosomes were first discovered and characterized by Christian de Duve and coworkers in the late 1950s and early 1960s (de Duve, 1959), as a result of studying the intracellular distribution of enzymes using centrifugal fractionation, cytological studies and biochemical analyses (de Duve et al., 1955). Due to his discovery de Duve was awarded the Nobel Prize in 1974 (de Duve, 1959; Duve, 1975). De Duve defined lysosome as a group of cytoplasmic particles surrounded by a lipoprotein membrane that contain a variety of acid hydrolases (de Duve and Wattiaux, 1966). Lysosomes are present in almost all cell types and their number depending on cell type and function (Wevers and Gieselmann, 2005).

Lysosomes are ubiquitous acidic vacuoles containing more than 50 different soluble lysosomal hydrolases including, glycosidases, proteases, lipases, nucleases, phospholipases, phosphatases, and sulfatases, that have acid pH optima and possess a major role in degradation of macromolecules from the secretory, endocytic, autophagic and phagocytic membrane-trafficking pathways (Wevers and Gieselmann, 2005; Luzio et al., 2007).

In addition to these acid hydrolases there are over 120 lysosomal membrane proteins. Lysosomal associated membrane protein type 1 (LAMP-1), lysosomal associated membrane protein type 2 (LAMP-2) and lysosomal integral membrane protein type 2 (LIMP-2) are the most abundant, and some of these membrane proteins are responsible for the transport of the other soluble enzymes and non-enzymes proteins in mannose 6-phosphate-independent transport (Braulke and Bonifacino, 2009).

During the past decade much has been discovered about many aspects of lysosome biogenesis. The selective phosphorylation of mannose residues on lysosomal enzymes, in conjunction with specific receptors for the mannose 6-phosphate (M6P) recognition marker, is found to be largely responsible for the targeting or "sorting" of newly synthesized lysosomal enzymes (Kornfeld and Mellman, 1989).

1.1. Ultra-Structure

Lysosomes were initially discovered by the biochemical fractionation of cell extracts and only later they were seen clearly by electron microscopy (Alberts et al., 2002). By electron microscopy studies, De Duve and co-workers demonstrated that lysosomes constitute up to 5% of the intracellular, are of heterogeneous size and morphology, and can often contain electron-dense deposits (Luzio et al., 2007). Lysosomes can be described as “dense bodies”, that are delimited by a single membrane and having an electron-dense content (Holtzman, 1989). They assume so many different forms additional to the dense bodies, reason why they cannot be recognized by the canonical morphological criteria of size, shape and internal structure, since these organelles vary significantly (Duve, 1983). They display considerable structural heterogeneity and appear in all shapes, sizes and densities (Wevers and Gieselmann, 2005). Lysosomes (Figure 1) are characteristically observed by electron microscopy as organelles of ~0.5 μm diameter and often have electron-dense cores. One marker used to identify this organelle was acid phosphatase (Holtzman, 1989). This enzyme can be identified histochemically and, thus, used in electron microscopy techniques (Weissmann, 1964). Morphologically, conventional lysosome is multi-vesicular in structure, as it contains internal vesicles that have budded off from the limiting membrane in a similar manner to a late endosome or multi-vesicular body (MVB) (Blott and Griffiths, 2002).

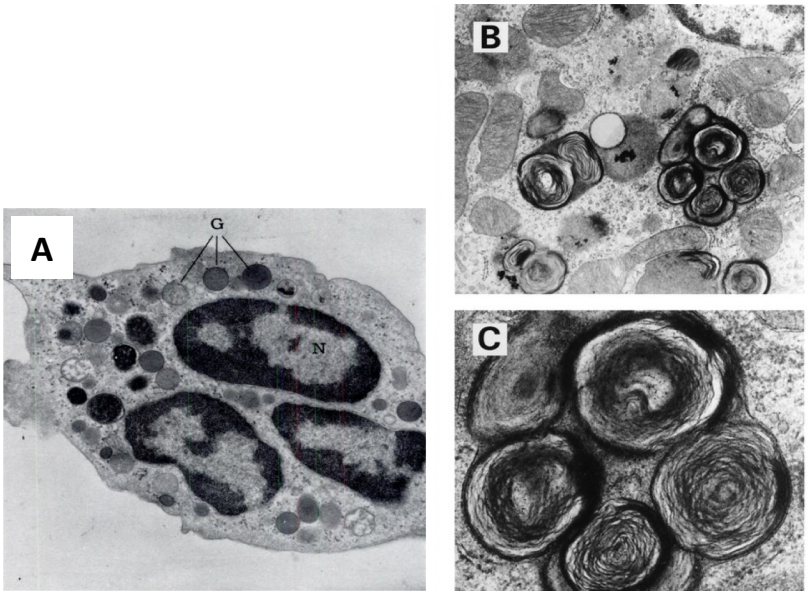

Figure 1- A- Electron micrograph of lysosomes. G-Lysosomes; N-Nucleus (Weissman, 1964). B and C correspond to a multilamellar structure of lysosomes (Taken from Ohshima et al., 1997).

1.2. Biogenesis

Lysosomal enzymes, along with secretory proteins and PM proteins, are synthesized on membrane-bound polyribosomes on the rough endoplasmic reticulum (RER) (Wevers and Gieselmann, 2005; Ni et al., 2006). After their synthesis, lysosomal proteins are sorted initially via signal peptides into the lumen of the endoplasmic reticulum (ER) (Wevers and Gieselmann, 2005). In this organelle lysosomal enzymes undergo glycosylation. This step involves the transfer of large oligosaccharide residues of the nascent protein (Wevers and Gieselmann, 2005). Then the signal peptide is cleaved, the protein folds, and the processing of the asparagine-linked oligosaccharide begins (Wevers and Gieselmann, 2005). Upon exit from the ER the *N*-glycosylated lysosomal pro-hydrolases travel via vesicular transport to the Golgi apparatus to acquire the M6P group (Castino and Isidoro, 2008). So these pro-hydrolases carry a unique marker in the form of M6P groups, which are added exclusively to the *N*-linked oligosaccharides of these soluble lysosomal enzymes as they pass through the lumen of the *cis* Golgi network (Alberts et al., 2002). In the *cis* compartment of the Golgi complex, oligosaccharide side chains of lysosomal enzymes are phosphorylated and thus acquire M6P moieties (Wevers and Gieselmann, 2005). These reactions occur in distinct compartments along the secretory pathway (Castino and Isidoro, 2008).

The synthesis of the M6P residues is initiated by a phosphotransferase, witch specifically recognizes lysosomal enzymes (Wevers and Gieselmann, 2005). This recognition is mediated by spatial signal depending on the three-dimensional structure of the enzymes. Only surface-located lysine residues seem to be an essential common component of this topogenic signal (Alberts et al., 2002). The M6P groups are recognized by transmembrane mannose 6-phosphate receptor (MPRs) proteins, which are present in the *trans* Golgi network (TGN). The receptor proteins bind to lysosomal hydrolases on the luminal side of the membrane and to adaptins in assembling clathrin coats on the cytosolic side (Figure 2) (Alberts et al., 2002). In this way, they help package the hydrolases into clathrin-coated vesicles that bud from the *trans* Golgi network. The vesicles subsequently deliver their contents to a late endosome (Alberts et al., 2002). The two-MPRs were identified because of their ability to bind M6P-containing soluble acid hydrolases in the Golgi and transport them to the endosomal-lysosomal system (Wevers and Gieselmann, 2005).

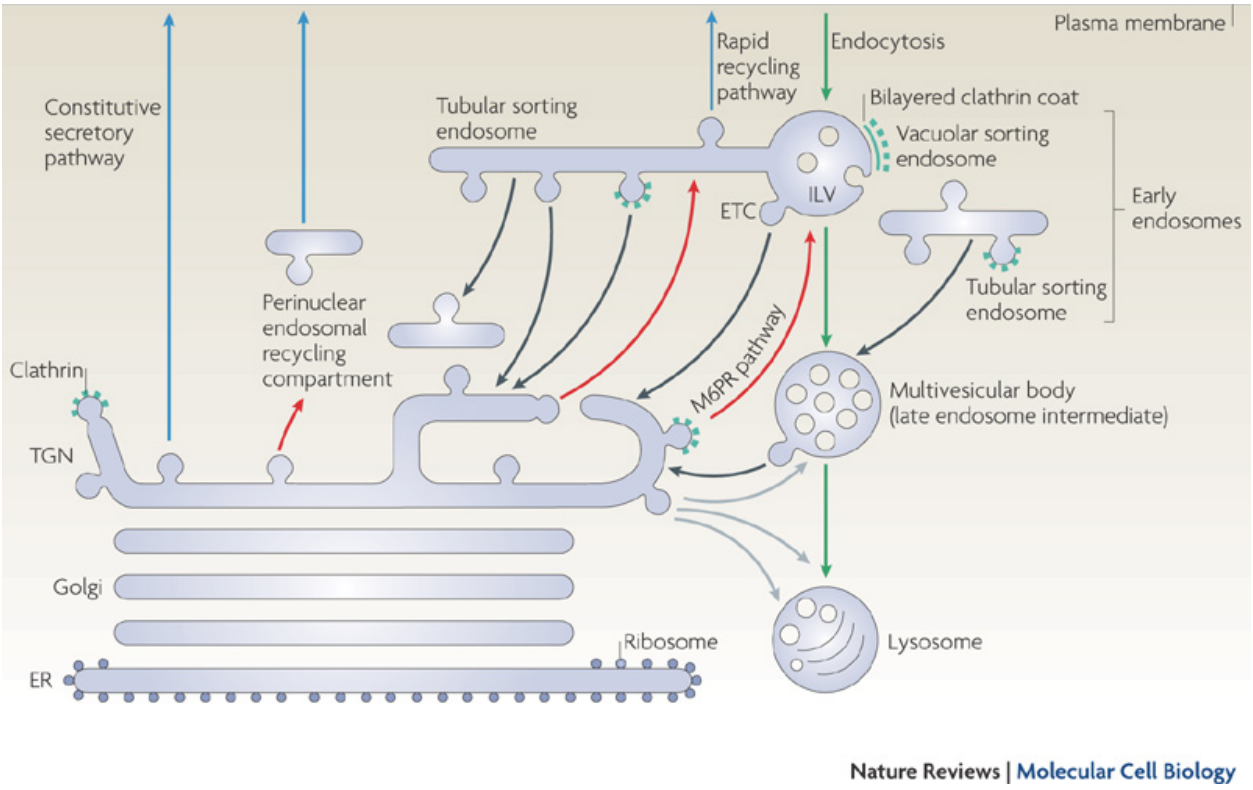

Figure 2 - Model for lysosomal enzyme targeting to lysosomes.

Lysosomal hydrolases and secretory proteins are synthesized in the RER and transported to the Golgi where the lysosomal enzymes acquire phosphomanosytol residues. Lysosomal biogenesis involves biosynthetic and endocytic pathways (green arrows). The soluble lysosomal proteins leave the ER, where they are synthesized, by vesicular transport passing through the ER-Golgi intermediate compartment. From the TGN, they can follow the constitutive secretory pathway (blue arrows) to the PM and subsequently reach lysosomes by endocytosis. Lysosomal proteins can go straight to the intracellular pathway (red arrows) to the endo-lysosomal system. Clathrin-dependent transport of lysosomal hydrolases is implicated in MPRs. Grey arrows represent endo-lysosomal pathways at distinct stages of maturation. The black arrows represent multiple retrograde pathways from endosomes (Taken from Saftig and Klumperman, 2009).

The bound hydrolases are first delivered to endosomes, where they dissociate from the receptors as a result of the acidic luminal pH. Lysosomes can be distinguished from endosomes by the absence of MPRs. This allows the receptors to recycle back to the Golgi apparatus and the hydrolases continue the transport to the lysosomes (Luzio et al., 2000). Unlike soluble hydrolases, the delivery of newly synthesized lysosomal membrane proteins from the Golgi complex does not require their binding to MPRs, and occurs either by an indirect route via the PM or by a direct intracellular route (Luzio et al., 2007).

In mammalian cells, the majority of soluble acid hydrolases are modified with M6P residues, allowing their recognition by MPRs in the Golgi complex and ensuing transport to the endosomal/lysosomal system. However, in addition to this M6P transport route, other soluble enzymes and non-enzymatic proteins are transported to lysosomes in an

M6P-independent manner mediated by alternative receptors such as the lysosomal integral membrane protein sortilin or LIMP-2 (Lefrancois et al., 2003; Mari et al., 2008). Sortilin is a protein with 100 KDa and belongs to a growing family of multiligand type-1 (Lefrancois et al., 2003; Westergaard et al., 2004). This transporter has been reported to mediate lysosomal trafficking of prosaposin and acid sphingomyelinase (ASMase) (Braulke and Bonifacino, 2009). Recently, the LIMP-2, a heavily N-glycosylated type III transmembrane protein, has been identified as a specific receptor for lysosomal targeting of β-glucocerebrosidase in an M6P-independent manner (Braulke and Bonifacino, 2009). The complex interaction of both luminal and cytosolic signals with recognition proteins guarantees the specific and directed transport of proteins to lysosomes (Braulke and Bonifacino, 2009).

1.3. Function

Lysosomes are membrane-bound organelles that are present in virtually all eukaryotic cells. They represent the final destination for a significant fraction of all intracellular traffic, and is one of the most important intracellular mechanisms for the turnover of endogenous and exogenous macromolecules (Kornfeld and Mellman, 1989). The catabolism occurs in the lumen of lysosomes by a combination of predominantly hydrolytic enzymes with acidic pH-optima ranging from 4.6–5.0, provided by proton-pumping vacuolar adenosine triphosphatases (ATPases) (Luzio et al., 2007). The lysosomal membrane separates the hydrolytic enzymes from the cytoplasm to protect against attack by the cell´s own digestive system (Alberts et al., 2002).

Lysosomes are acidic pH hydrolase-rich vacuoles and they are responsible for degradation of a variety of substances from the extracellular space by endocytosis, delivered by fusion with phagosomes or autophagosomes, or derived from the biosynthetic pathway, such as proteins, glycosaminoglycans, nucleic acids, oligosaccharides, and complex lipids, into their building blocks that are available for metabolic re-use (Luzio et al., 2000; 2007).

These compartments eventually fuse with primary lysosomes to form various types of secondary lysosomes, and can include autophagic vacuoles, multilamellar bodies (MLB), MVB, and dense bodies (Nixon and Cataldo, 1993) (Figure 3). These secondary lysosomes can reflect the nature of the internalized material and may indicate the states in

the digestion process and can be identify by electron microscopy (de Duve and Wattiaux, 1966). Lysosomes are distinguished from endosomes by the absence of the two MPRs and recycling cell surface receptors (Luzio et al., 2000).

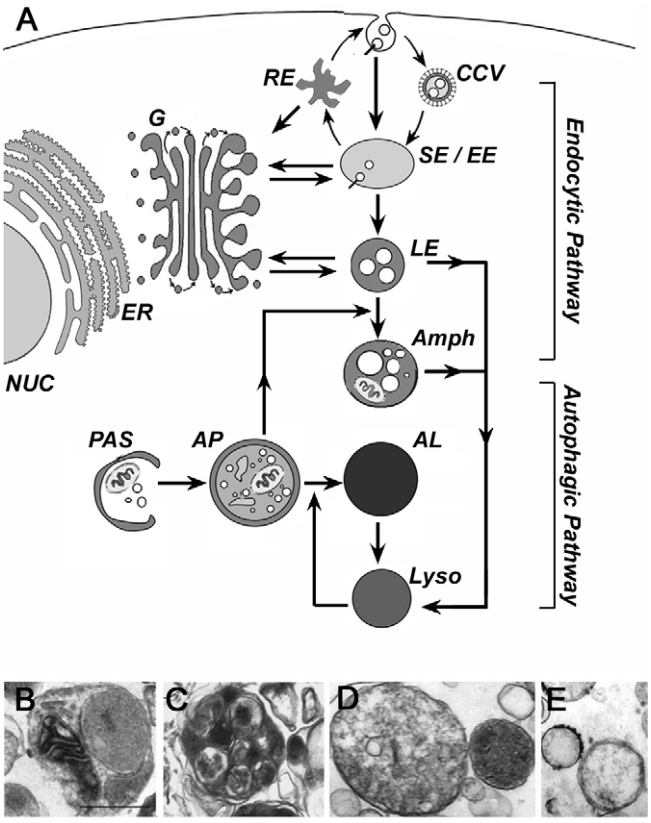

Figure 3- Scheme of the lysosomal system representing endocytic and autophagic pathways. (B-E) ultrastructure of the lysosome

A -. Pre-autophagic structure (PAS) sequesters large areas of cytoplasm within a double membrane-limited autophagosome (AP). This organelle fuses with a lysosome to form an autophagolysosome (AL) or with a late endosome/multivesicular body (LE/MVB) to form an amphisome. (B) Internalized materials entering the endocytic pathway are directed to early (sorting) endosomes (EE), which mature to LE/MVB. (C) An autophagosome, which is hydrolase- negative, contains recognizable but partially digested organelle compartments. (D) Another type of immature autophagic vacuoles (AV) is double-membrane limited and contains heterogeneous intraluminal materials, including other organelles. (E) Following further substrate digestion, the content of an autolysosomes is amorphous and less dense. Complete digestion of substrates within autolysosomes ultimately yields lysosomes, which are smaller, less dense vesicles containing mainly lysosomal hydrolases (E). Bar, 500 nm (Taken from Nixon, 2007).

The mechanism of transport of the endocytosed material from endosomes to lysosomes remains controversial, and several theories were proposed (Luzio et al., 2007). Three alternative hypotheses are available. These include: (i) Maturation of the endosome into a lysosome, that involves formation of EE by coalescence of vesicles from the PM; (ii) Vesicular transport with vesicles carrying cargo from endosomes to lysosomes,

postulating that EE, LE and lysosomes are stable compartments; (iii) Kiss-and-run, a continuous repeated cycles of fusion and fission, during which material is transferred between the organelles and each contact is followed by a dissociation or 'run' (Storrie and Desjardins, 1996; Luzio et al., 2000; 2007). A recently proposed variation of "kiss-and-run", direct fusion, in which lysosomes reform from hybrid organelles as a result of fission events (de Duve et al., 1955; Luzio et al., 2000; 2007). These models are not mutually exclusive and it is possible that cells employ more than one of these processes in lysosomal biogenesis (de Duve, 1959; Duve, 1975; Luzio et al., 2000; 2007).

Lysosomes used to be considered just as a degradation organelle (Figure 4). However, recent studies of lysosomes and their proteins in association with endosomal, autophagosomal, and related vesicular systems, have re-conceptualized lysosomes as vital recycling centers, influencing signal transduction and regulation of gene expression. The Transcription Factor EB (TFEB) positively regulates these key processes by regulating the Coordinated Lysosomal Expression and Regulation (CLEAR) network. Genes that belong to this pathway are involved in lysosomal biogenesis, exocytosis and autophagy (Settembre et al., 2013).

Lysosomes play critical roles in other cellular processes. They also fuse with the PM during cell injury, as well as having more specialized secretory functions in some cell types (de Duve and Wattiaux, 1966; Luzio et al., 2007). Not so far was fond another important function for lysosome, related to sensing and signaling pathways. This complex mechanism can sense nutrient availability and activate a lysosome-to-nucleus signaling pathway that is involved in cell metabolism and growth (Wevers and Gieselmann, 2005; Settembre et al., 2013). In addition, recent studies suggest new lysosomal function, they can be involved in membrane repair (Wevers and Gieselmann, 2005; Luzio et al., 2007).

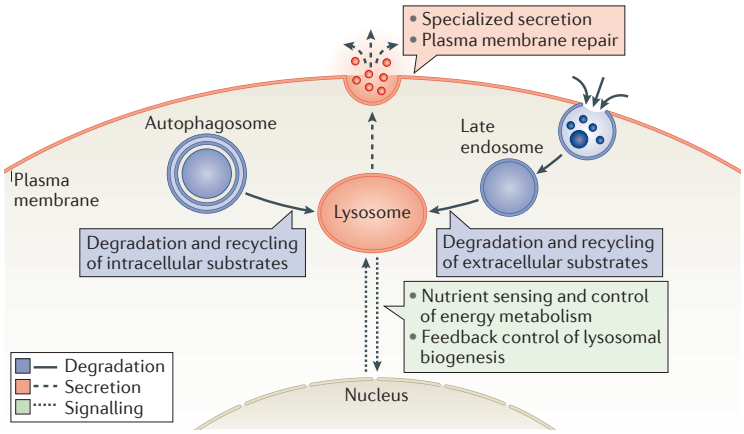

Figure 4- Main functions of the lysosome.

Lysosomes can be involved in many functions such as, degradation and recycling of intracellular substrates; specialized secretion; PM repair; degradation and recycling of extracellular substrates; nutrient sensing and control of energy metabolism; feedback control of lysosomal biogenesis (Taken from Settembre et al., 2013)

2. Sphingolipids

The prefix sphingo derives from the Greek myth of the sphinx and signifies the magnitude of the riddle associated with the function of these molecules (Kornfeld and Mellman, 1989; Nyberg et al., 1998). Sphingolipids (SLs) represent a major class of lipids that are ubiquitous constituents of membranes in all animals. However they can also be found in plants, fungi, as well as some prokaryotic organisms and viruses (Alfred H Merrill and Hannun, 2000; Alberts et al., 2002; Bartke and Hannun, 2009).

More than 300 different types of complex SLs have been reported, in various mammalian cell types (Hannun and Bell, 1989; Alfred H Merrill and Hannun, 2000; Luzio et al., 2007). Many authors contributed to their discovery, but one can be considered the father of sphingolipid (SL) research. In 1884 Johann L. W. Tudichum published their findings in a book entitled *A Treatise on the Chemical Constitution of the Brain* (Holtzman, 1989; Yamakawa, 1996; Alfred H Merrill and Hannun, 2000; Anon, 2000; Merrill and Sandhoff, 2002). He was a German clinician with training in chemistry and isolated an organic base that he called sphingosine (Duve, 1983; Yamakawa, 1996). He also isolated and named several lipids such as cerebroside, Cer, sphingomyelin (SM), and cephalin from human brain (Thudichum, 1884; Wevers and Gieselmann, 2005).

2.1. Structure

SLs are amphipathic molecules that have both hydrophobic and hydrophilic properties, consisting of a long chain base, usually sphinganine or sphingosine, attached to a fatty acid via an amide bond. The hydrophobic region consists of a sphingoid long chain base (normally sphingosine, sphinganine or phytosphingosine) to which a fatty acid is attached by an amide bond to carbon 2 (Holtzman, 1989; Merrill et al., 2001; Merrill and Sandhoff, 2002; Futerman and Hannun, 2004; Kacher and Futerman, 2006) (Figure 5). Sphingosine is the prevalent backbone of mammalian SLs and is sometimes used as a generic term for all sphingoid bases (Thudichum, 1884; Weissmann, 1964; Merrill et al., 2001).

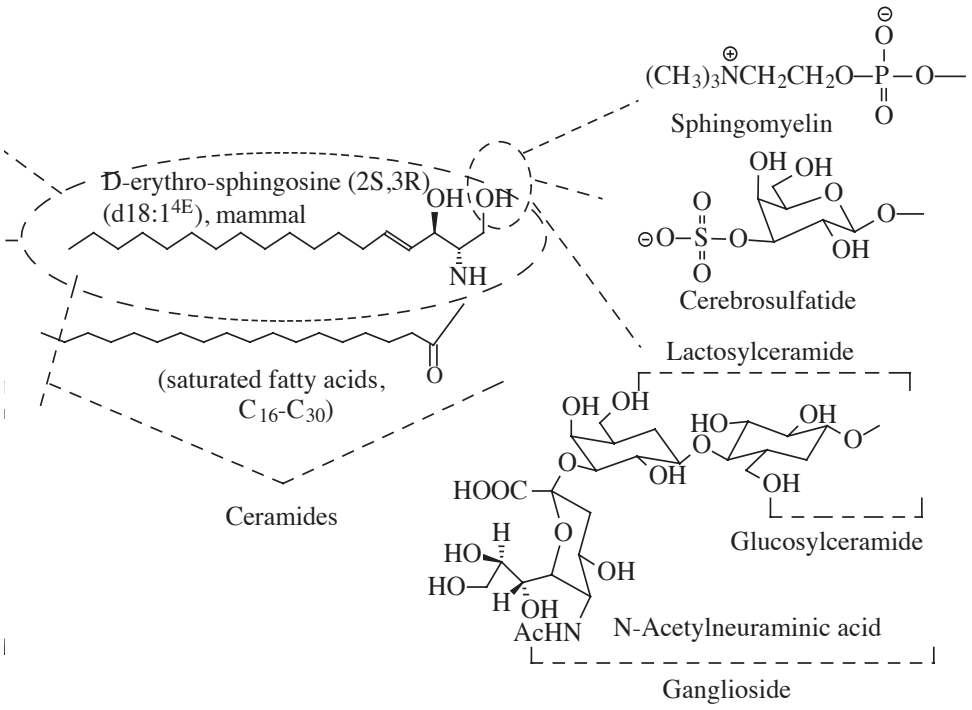

Figure 5- Chemical structures of SLs

SLs are amphipathic molecules having both hydrophobic and hydrophilic properties, consisting of a long chain base, usually sphinganine or sphingosine, attached to a fatty acid via an amide bond, and usually contain different head groups. Cer, the backbone of all SLs, is composed of a long chain base acylated with a fatty acid. A major constituent of cell membranes, SM, comprises a Cer unit and a phosphorylcholine or phosphoethanolamine polar headgroup. SLs can have a sugar head groups that can vary in complexity from a single glucose (glucosylceramide) or galactose (galactosylceramide), in the case of cerebrosides. To more complex structures, such as lactosylceramide with two sugars. Certain sub-clases are recognized by additional components, such as sulfatides, which contain sulfate, and gangliosides, which contain sialic acid residues (Taken from Guo et al., 2005).

Cer (N-acyl-sphingosine) is the backbone of all SLs, which are essential constituents of eukaryotic membranes. This simple SL is composed of a fatty acid attached to a long chain base via an amide bond. The composition of the fatty acids in Cer vary in length, typically 14 to 24 carbon atoms, and degree of saturation, normally being saturated or mono unsaturated and might also contain a hydroxyl group on the second carbon (Futerman and Hannun, 2004). Sphingosine, the most abundant long chain base in mammals, is composed by 18-carbons with a double bond at the 4-5 position. On the other hand, sphinganine, that is the product of *de novo* synthesis, lacks this double bond. Other long chain bases may have different number of carbons (14-22 carbons), hydroxyl groups (mainly at positions 4 and 6), more than one double bond and even branching of a methyl group on carbon 9 (Weissmann, 1964; Merrill and Sandhoff, 2002).

Glycosphingolipids (GSLs) are composed of a Cer backbone and a sugar headgroup. Only the eukaryotes and a few bacteria contain these heterogeneous class of lipids (Degroote et al., 2004; Wevers and Gieselmann, 2005; Ni et al., 2006). It is known that the carbohydrate residue is attached by a glycosidic linkage to O-1 of the sphingoid. GSLs on the cell surface are involved in cell-type specific adhesion processes and can function as binding sites for toxins, viruses and bacteria (Kolter and Sandhoff, 1999; Wevers and Gieselmann, 2005).

2.2. Metabolism and transport

The metabolic pathway of SLs represents a complex network of reactions in which the central molecule is Cer (Figure 6). This includes: the biosynthesis of Cer and GSLs that occurs in the ER and Golgi complex, and sequential degradation of GSLs to Cer by the action of specific exohydrolase in the lysosome (Wevers and Gieselmann, 2005; Kitatani et al., 2008). The lysosomal Cer is subsequently converted into sphingosine and fatty acid by the action of ACDase. Sphingosine can be transported through the lysosomal membrane and be used in the enzymatic conversion to Cer or sphingosine-1-phosphate (S1P). Additionally, Cer can be formed from SM by sphingomyelinase action. In the case of ASMase, the enzyme is present in endosomes/lysosomes of all cells (Wevers and Gieselmann, 2005; Kitatani et al., 2008).

Pathways of SL metabolism have a unique metabolic entry point, serine palmitoyl transferase (SPT), which forms the first SL in the *de novo* pathway, and a unique exit

point, S1P lyase, which breaks down S1P into non-SL molecules (Castino and Isidoro, 2008; Hannun and Obeid, 2008).

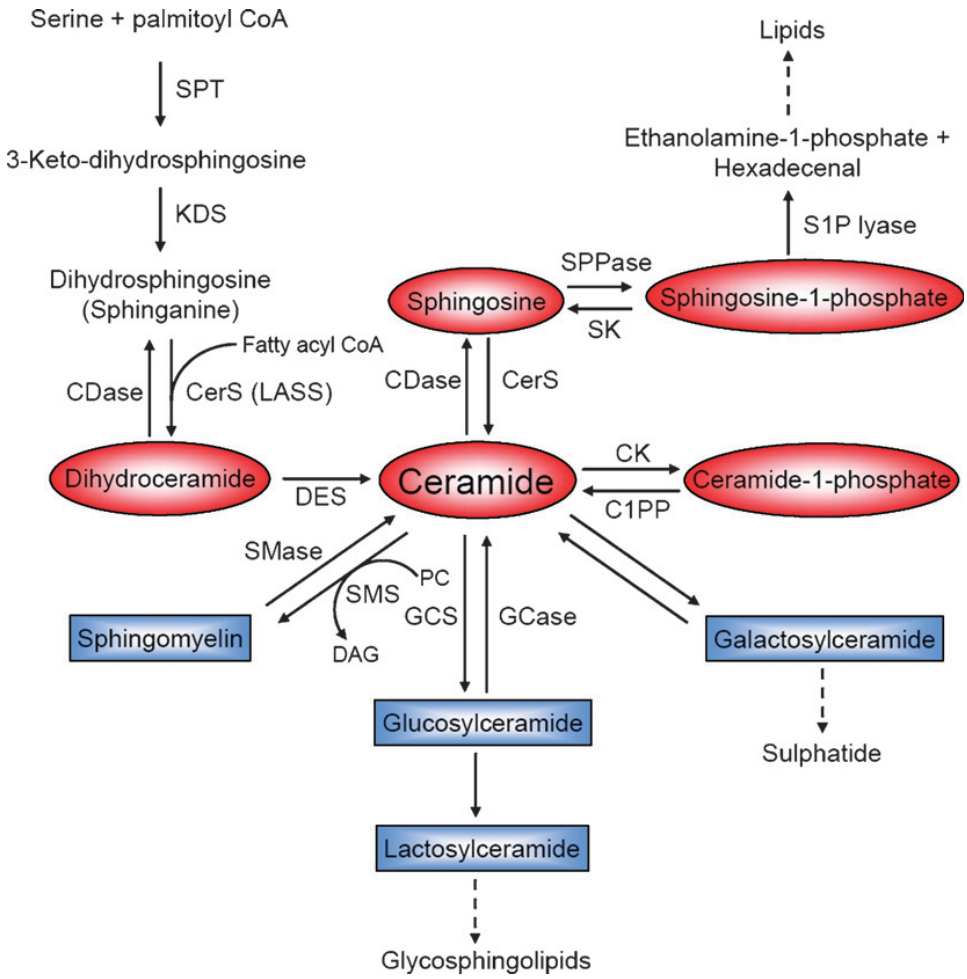

Figure 6 - The central position of Cer in SL metabolism

Cer can come from de novo synthesis due to stimulation of SPT and/or dihydroceramide synthase, or by degradation of sphingomyelins via spingomyelinases. It can also be generated through metabolism of complex SLs. A major metabolite of ceramide is ceramide-1-phosphate (C1P), which can be formed through direct phosphorylation of ceramide by ceramide kinase. The reverse reaction is catalyzed by ceramide-1-phosphate phosphatase, or by lipid phosphate phosphatases. There is increasing evidence suggesting that C1P can regulate cell proliferation and apoptosis. Alternatively, Cer can be degraded by ceramidases to form sphingosine, which can, in turn, be phosphorylated to S1P by sphingosine kinases. The reverse reaction is catalyzed by S1P phosphatases, or by lipid phosphate phosphatases. SM N-deacylase generates sphingosylphosphorylcholine. Bioactive SL metabolites are highlighted in red. SPT, serine palmitoyl transferase; KDS, 3-keto-dihydrosphingosine reductase; DES, dihydroceramide desaturase; SPPase, Sph phosphate phosphatase; CK, Cer kinase; C1PP, C1P phosphatase; SMS, SM synthase; PC, phosphatidylcholine; DAG, diacylglycerol; GCS, glucosylceramide synthase; GCase, glucosyl CDase. (Taken from Bartke and Hannun, 2009)

2.3. Function

As mentioned above, SLs are found in essentially all animals, plants, and fungi, as well as some prokaryotic organisms and viruses. They are mostly in membranes, but are also major constituents of lipoproteins. The functions of SLs are still being discovered, but there are at least three, i.e., structure, recognition and signal transduction (Merrill and Sandhoff, 2002; Wevers and Gieselmann, 2005). Besides the role in membrane and lipoprotein structure, they also act in cell regulation as second messengers for growth factors, differentiation factors and cytokine. SLs have also been implicated in intracellular signaling, modulating a variety of events such as cell-cell interaction, proliferation, differentiation, cell death, and stress responses (Futerman and Hannun, 2004; Kacher and Futerman, 2006; Castino and Isidoro, 2008).

3. Lysosomal Storage Disorders: Sphingolipidoses

Was Hers who introduced in 1965 the concept of "lysosomal storage disorders" (LDSs) to explain how genetically determined absence of a seemingly unimportant enzyme, α-glucosidase, could lead to the fatal condition known as Pompe disease (Hers, 1965; Neufeld, 1991; Wevers and Gieselmann, 2005). After this, a series of important discoveries were made, regarding intracellular biology of these enzymes and their substrates, that culminated on the successful treatment of Gaucher's disease through enzyme replacement therapy with β-glucosidase in the early 1990s (Jmoudiak and Futerman, 2005).

LSDs, of which more than 50 are known (Table 1), are a heterogeneous group of inherited metabolic neurodegenerative disorders due to deficiency of a specific protein required for lysosomal function, such as enzymes or lysosomal components, or to errors in enzyme trafficking/targeting that are involved in lysosomal biogenesis or protein maturation, all preventing the complete degradation and recycling of macromolecules, which results in the intra-lysosomal accumulation of undegraded metabolites (Alberts et al., 2002; Futerman and Van Meer, 2004; Mehta et al., 2006; Bellettato and Scarpa, 2010). This is determined by the specific mutation(s) that an individual carries and the effect of that specific mutant genotype on enzyme stability and/or function. The majority of LSDs are inherited in an autosomal recessive fashion, except for three disorders: Fabry disease, Dannon disease, and Hunter syndrome (Mucopolisacaridosis type II) that are all X-linked recessive (Alberts et al., 2002; Greiner-Tollersrud and Berg, 2005; Pastores,

2011). Almost invariably, heterozygotes with 50% enzymatic activity do not develop the disease, a notable exception being some female carriers of the X-linked disorder Fabry disease (Jeyakumar et al., 2005; Wevers and Gieselmann, 2005).

The importance of a physiologically appropriate SL catabolism is demonstrated by the severity of sphingolipidoses. In these diseases, mutations in genes encoding acid hydrolases or protein cofactors, cause a blockage in specific steps of the lysosomal degradation pathway of SLs (Futerman and Van Meer, 2004; Saftig and Klumperman, 2009).

2. Table 1 – Some of LSDs (Taken from Futerman and van Meer, 2004)

Disease	Defective protein	Main storage materials
Sphingolipidoses		
Fabry	α-Galactosidase A	Globotriasylceramide and blood-group-B substances
Farber lipogranulomatosis	Ceramidase	Ceramide
Gaucher	β-Glucosidase Saposin-C activator	Glucosylceramide Glucosylceramide
Niemann–Pick A and B	Sphingomyelinase	Sphingomyelin
Sphingolipid-activator deficiency	Sphingolipid activator	Glycolipids
GM1 gangliosidosis	β-Galactosidase	GM1 ganglioside
GM2 gangliosidosis (Tay–Sachs)	β-Hexosaminidase A	GM2 ganglioside and related glycolipids
GM2 gangliosidosis (Sandhoff)	β-Hexosaminidase A and B	GM2 ganglioside and related glycolipids
GM2 gangliosidosis (GM2-activator deficiency)	GM2-activator protein	GM2 ganglioside and related glycolipids
Mucopolysaccharidoses (MPS)		
MPS I (Hurler, Scheie, Hurler/Scheie)	α-Iduronidase	Dermatan sulphate and heparan sulphate
MPS II (Hunter)	Iduronate-2-sulphatase	Dermatan sulphate and heparan sulphate
MPS IIIA (Sanfilippo)	Heparan *N*-sulphatase (sulphamidase)	Heparan sulphate
MPS IIIB (Sanfilippo)	*N*-Acetyl-α-glucosaminidase	Heparan sulphate
MPS IIIC (Sanfilippo)	Acetyl-CoA:α-glucosamide *N*-acetyltransferase	Heparan sulphate
MPS IIID (Sanfilippo)	*N*-Acetylglucosamine-6-sulphatase	Heparan sulphate
Morquio-A disease	*N*-Acetylgalactosamine -6-sulphate-sulphatase	Keratan sulphate, chondroitin-6-sulphate
Morquio-B disease	β-Galactosidase	Keratan sulphate
MPS VI (Maroteaux–Lamy)	*N*-Acetylgalactosamine-4-sulphatase (arylsulphatase B)	Dermatan sulphate
MPS VII (Sly)	β-Glucuronidase	Heparan sulphate, dermatan sulphate, chondroitin-4- and -6-sulphates
Oligosaccharidoses and glycoproteinosis		
Pompe (glycogen-storage-disease type II)	α-Glucosidase	Glycogen
Diseases caused by defects in integral membrane proteins		
Cystinosis	Cystinosin	Cystine
Danon disease	LAMP2	Cytoplasmic debris and glycogen
Infantile sialic-acid-storage disease and Salla disease	Sialin	Sialic acid
Mucoplipidosis (ML) IV	Mucolipin-1	Lipids and acid mucopolysaccharides
Niemann–Pick C (NPC)	NPC1 and 2[‡]	Cholesterol and sphingolipids
Others		
Galactosialidosis	Cathepsin A	Sialyloligosaccharides
I Cell and pseudo-Hurler polydystrophy (ML II and ML III, respectively)[§]	UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosaminyl-1-phosphotransferase	Oligosaccharides, mucopolysaccharides and lipids
Multiple sulphatase deficiency	Cα-formylglycine-generating enzyme	Sulphatides
Neuronal ceroid lipofuscinosis (NCL)1 (Batten disease)[‖]	CLN1 (protein palmitoylthioesterase-1)	Lipidated thioesters
NCL2 (Batten disease)	CLN2 (tripeptidyl amino peptidase-1)	Subunit c of the mitochondrial ATP synthase
NCL3 (Batten disease)	Arginine transporter	Subunit c of the mitochondrial ATP synthase

Sphingolipidoses are a group of inherited disorders of lipid metabolism affecting the central nervous system (CNS). Mostly affecting pediatric population, these diseases are characterized by progressive involvement of neurons, with psychomotor retardation. However, this is a heterogeneous group of diseases and the diagnosis on clinical groups is oftentimes difficult. Niemann-Pick disease, Krabbe's disease, Gaucher's disease, Metachromatic leukodystrophy, Tay-Sachs disease, and generalized gangliosidosis, are examples of sphingolipidoses (Brady, 1978; Luzio et al., 2000). Sphingolipidoses are due to an impaired lysosomal digestion of GSLs metabolism. GSLs are degraded by the action of exohydrolases and in some cases by SL activator proteins. Until now there are five SL activator protein (SAPs), the SAP-A to D also called saposins and the GM2-activator (Kolter and Sandhoff, 1998; Luzio et al., 2007).

Even though, separately, these disorders are rare, when considered all together their prevalence is much higher, 1 in 5000 children will be found to have an LSD, caused primarily by a deficiency of a lysosomal hydrolase or its cofactor (Lefrancois et al., 2003; Mari et al., 2008; Pastores, 2011). Certain disorders have higher prevalence in specific geographic areas or among those of a particular ethnicity. In terms of geographic areas aspartylglucosaminuria and Salla disease, are particularly frequent in Finland; in terms of ethnicity, Gaucher and Tay-Sachs disease, are almost 100 times more prevalent in Ashkenazi Jewish descendant that in the general population (Lefrancois et al., 2003; Westergaard et al., 2004; Greiner-Tollersrud and Berg, 2005). In Portugal, for example, the overall birth prevalence of 29 different LSDs studied was calculated to be 25/100 000 live births, which is double the prevalence that has previously been described in Australia and in The Netherlands (Meikle et al., 1999; Poorthuis et al., 1999; Pinto et al., 2004; Braulke and Bonifacino, 2009).

3.1. Diagnosis

In sphingolipidoses a wide spectrum of clinical phenotypes is observed, with differences in the age of onset, clinical severity or neurological involvement, and typically has a neurodegenerative course (Beck, 2001; Jeyakumar et al., 2005; Braulke and Bonifacino, 2009). In general, neuropathological symptoms include developmental delay, abnormal ocular movements, ataxia, seizures, movement disorders, spasticity, visual loss and psychiatric disease (psychosis, depression and dementia). Although most sphingolipidoses have onset in childhood, several subtypes have later-onsets with symptoms that may not be evident until adulthood. One of the main factors that determine

the severity of a LSD, such as Sandhoff or Tay–Sachs disease, is the residual activity of the affected enzyme (Conzelmann and Sandhoff, 1983; Kornfeld and Mellman, 1989). Thus, the best known predictor of age of onset and clinical course of the diseases is the level of residual enzyme activity (Jeyakumar et al., 2005; Luzio et al., 2007). This is determined by the specific mutation(s) carried by each individual. The diagnosis in suspected cases can be confirmed by biochemical and/or molecular assays, which can be applied for prenatal and presymptomatic diagnosis (Alberts et al., 2002; Greiner-Tollersrud and Berg, 2005; Pastores, 2011). Early diagnosis and intervention before the onset of irreversible pathology will provide a substantial benefit to many of these newborns, as well as providing the opportunity for parents to receive genetic counseling. Thus, prevalence data are important to delineate prevention and therapeutic strategies (Luzio et al., 2000; Pinto et al., 2004; Luzio et al., 2007).

3.2. Molecular Pathogenesis

As mentioned above, a wide spectrum of clinical phenotypes is observed, with differences in the age of onset, clinical severity or neurological involvement, and typically has a neurodegenerative course (Nixon and Cataldo, 1993; Beck, 2001; Jeyakumar et al., 2005). In sphingolipidoses, as for all the other LSDs, the undegraded substrate gradually accumulates within lysosomes. However, it is now recognized that these disorders are not simply a consequence of pure storage, but result from perturbation of complex cell signaling mechanisms where the different mutations have different effects on the enzyme stability and/or function and, consequently, different biological outcome (de Duve and Wattiaux, 1966; Vellodi, 2005). The relationship between genotype and phenotype at the level of disease process remains poorly defined and, for most storage disorders, this is only an approximate guide to clinical outcome (Walkley, 1998; Luzio et al., 2000; Jeyakumar et al., 2005). The appearance of these diseases could presumably occur due to the defective activity of one or more of the enzymes involved in the degradative pathway, and consequently leading to changes in the balance between specific SL signaling molecules, causing effects on cell function and survival (Kolter and Sandhoff, 1999; Nixon, 2007) (Figure 7).

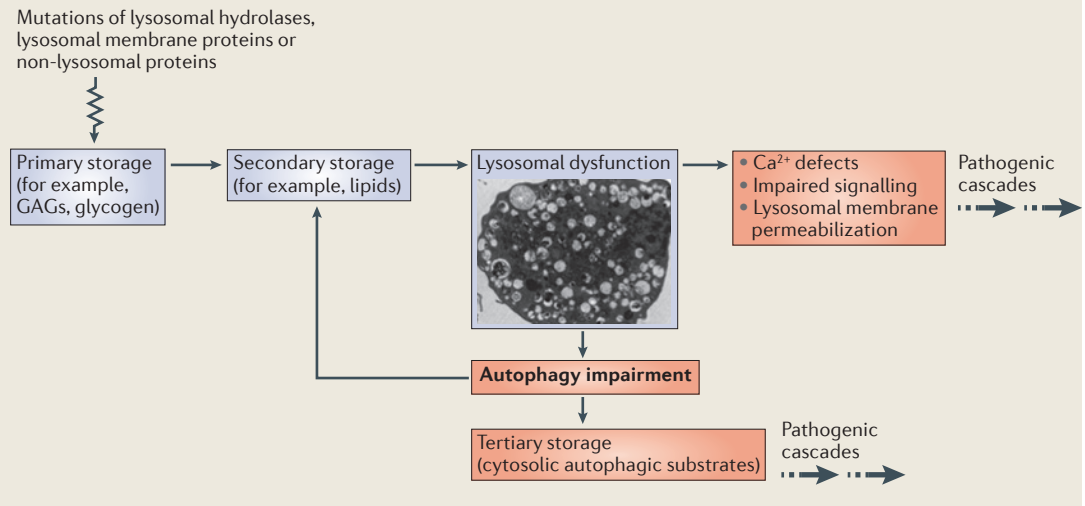

Figure 7 - Mechanism of LSDs (Taken from Settembre et al., 2013).

3.2.1 Apoptosis

Programmed cell death occurs primarily through an evolutionarily conserved form of cell suicide called apoptosis (Storrie and Desjardins, 1996; Vaux, 1998; Luzio et al., 2000; 2007). In apoptosis, a biochemical cascade activates proteases that destroy molecules that are required for cell survival and others that mediate a program of cell suicide (Friedlander, 2003). This event occurs In multicellular organisms and it is important for cell homeostasis maintained through a balance between cell proliferation and cell death (Vaux, 1998). Apoptosis can be induced in several cell types by tumor necrosis factor (TNF), anti-fas antibody, radiation and chemotherapeutic agents such as actinomycin D, through generation of Cer by activation of the sphingomyelinase pathway or ceramide synthase (Hannun, 1996; Tohyama et al., 1999). This simple SL can act as an intracellular mediator of cell growth, differentiation and apoptosis (Hannun, 1996; Tohyama et al., 1999). In the lysosome, ASMase induces Cer release (Hannun and Luberto, 2000). However, the mechanisms of ASMase/Cer-mediated death signaling are not completely clear. In stress conditions, such as defective activity of lysosomal proteins, which results in the intra-lysosomal accumulation of undegraded metabolites, the apoptotic cascade can be activated (Futerman and Van Meer, 2004). Previous work showed exacerbated apoptosis in cells or tissues from patients or animals affected with neurolipidoses, Tay–Sachs and Sandhoff (Tardy et al., 2004). Thus lysosomal dysfunction may affect

apoptosis, either stimulation or reduction through different potential mechanisms (Tardy et al., 2004).

Levade and co-workers (Tardy et al., 2004) enumerated some of this possible mechanisms:

1) Accumulation of a toxic compound, due to its deficient breakdown, galactosylsphingosine, which accumulates in Krabbe disease, is an example for this mechanism.
2) Lack of formation of a molecule acting as an apoptosis inducer; defect in the lysosomal hydrolase may result in an impaired formation of Cer in the acidic compartments or in the PM, this happens in Niemann–Pick disease (NPD).
3) Lack of a molecule acting as an apoptosis suppressor; an example is prosaposin that results in activation of mitogen-activated protein kinases leading to (pro)saposin deficiency, or CLN3, which attenuates Cer formation, leading to a form of juvenile neuronal ceroid lipofuscinosis (NCL).
4) Deficient activity of a protease in the lysosomal compartment; leading to the absence of proteolytic processing of a protein involved in apoptosis, it is caused by deficient activity of cathepsin C, which is the responsible for defective processing of granzymes, this syndrome is called Papillon–Lefèvre.
5) Deficient activity of a lysosomal protease that is potentially active outside the acidic compartments, cathepsins D or B may activate the caspase cascade.
6) Uncontrolled activation of lysosomal proteases and excessive proteolysis, due, for instance, to the lack of a cathepsin inhibitor. One example is cystatin B, a cysteine protease inhibitor; when this gene is mutated causes Unverricht–Lundborg disease.
7) Abnormal signal transduction of apoptotic signals due to a general perturbation of intracellular trafficking related to dysfunction of lysosomal catabolism. This situation could happen in cells of mucolipidosis type II and III.

Many reports indicate that Cer generation by sphingomyelinase is an important physiological cause of apoptosis (Tohyama et al., 1999). However some controversial observations have been reported regarding apoptosis induction in cells or tissues from NPD or FD. ASMase deficiency has been associated with resistance, in certain cell types, to programmed cell death triggered by ionizing or ultraviolet radiation, TNF, doxorubicin, or CD95 ligation (Peña et al., 1997; Canals et al., 2011), while defective in ACDase appeared to be linked with an increased apoptosis in colon (Canals et al., 2011). Other rare lysosomal disorders characterized by multiple substrate storage, are mucolipidoses.

Previous studies indicated that apoptosis is disturbed in these diseased cells. According to these findings that support the idea that lysosomal components are involved in apoptosis, in the presence of lysosomal dysfunction, cell-death program is compromised (Tardy et al., 2004).

3.2.2 Inflammation

Inflammation can be defined as an active defense reaction of multicellular organisms against diverse insults, designed to remove or inactivate noxious agents and to inhibit and reverse their detrimental effects (Wyss-Coray and Mucke, 2002). The catabolism of macromolecules in the lysosome is essential for the correct function of several immune system functions including antigen processing and presentation, cytokine secretion, phagocytosis, and secretion of molecules (Castaneda et al., 2008). The inflammation can results as a consequence of storage material disturbs in cellular function (Beck, 2010). Previous studies showed abnormal inflammatory responses in the brain in LSDs, such as, in glycosphingolipidoses, gangliosidoses, Sandhoff disease, and in all types of NCLs (Castaneda et al., 2008). The mechanisms leading to immune activation are not completely known, but probably reflect altered signaling pathways in response to storage (Vitner et al., 2010). In LSDs with CNS involvement, brain inflammation is a common feature (Smith et al., 2009; Vitner et al., 2010). After substrate accumulation in LSDs, an inflammatory response is triggered that is not self-limiting, and once triggered, it progressively increases in parallel with the storage burden (Vitner et al., 2010). Previous studies showed in the CNS of a Sandhoff disease mouse model and in a Sandhoff disease human autopsy, macrophage/ microglial activation, which was implicated in neuro-inflammatory component in this disorder (Jeyakumar et al., 2003). In GM1 and GM2 gangliosidoses, which store GM1 and GM2 ganglioside respectively, the activation of both CNS and peripheral inflammation predates the onset of clinical signs and involves elevation of multiple proinflammatory cytokines (Vitner et al., 2010). Thus, lysosomal storage causes immune activation by different molecular mechanisms depending on where storage occurs and on the biochemical nature of the stored molecules (Vitner et al., 2010). This allows the relationship between degree of inflammation, storage levels and pathology to be correlated (Jeyakumar et al., 2003). Once neuro-inflammation can be a key player in the pathogenic process, effective therapies in multiple LSDs would be predicted to impact this process and delay inflammation (Jeyakumar et al., 2003). One example was a mouse model of Sandhoff disease treated with non-steroidal anti-inflammatory drugs or crossed with the MIP1 alpha knock-out mouse to prevent peripheral

immune cell recruitment to the brain, resulting in clinical benefit. This benefit was also observed for mouse model of NPC1 (Vitner et al., 2010).

3.2.3 Autophagy

Cell death is usual classified into two groups, necrosis, a passive, uncontrolled way to die, and programmed cell death, which consists in a highly regulated process with defined cellular pathways. Programmed cell death was categorized into three types, according to the basis of morphological features, they include, the type 1 which is apoptosis; type 2, autophagy; and finally type 3, that is a nonlysosomal cell death. Autophagic cell death it has often been proposed to be an alternative mechanism of programmed cell death (Debnath et al., 2005).

The autophagic process was first described in mammalian cells many years ago (Baba et al., 1994) It represents an evolutionary mechanism conserved in eukaryotic cells where the lysosomal degradation pathway for cytoplasmic material is preceded by the formation of an AP, enclosed within a double membrane that engulfs part of the cytoplasm by fusing with endocytic compartments and lysosomes (Codogno and Meijer, 2005; Kiselyov et al., 2007). The latter ones are essential for maturation and completion of autophagy-initiated protein and organelle degradation (Kiselyov et al., 2007). This ubiquitous process can be stimulated in response to stress conditions, allowing the cell to adapt to environmental and/or developmental changes (Klionsky, 2007).

Three general types of autophagic processes have been identified in eukaryotic cells, named macroautophagy, microautophagy and chaperone-mediated autophagy. However the best-studied of these pathways is macroautophagy, which is linked to a range of human disease (Huang and Klionsky, 2007). Recent research suggests the defect in autophagic process may be a characteristic of a group of neurodegenerative diseases referred to as LSDs (Winslow and Rubinsztein, 2008). Without this fusion event APs accumulate with undegraded cargo, thus increasing the toxic species within cells (Winslow and Rubinsztein, 2008). Autophagy has been analyzed in a variety of LSDs with different phenotypic severities, different tissues involved, and different types of storage material. Previous studies suggested that an accumulation of one or more GSLs may alter the function of the autophagic pathway in these disorders (Lieberman et al., 2012). Another study suggested that lysosomal deficiencies in LSDs inhibit autophagic maturation, leading to a condition of autophagic stress (Kiselyov et al., 2007). Thus, a common factor can be the impairment of autophagy and accumulation of autophagy

substrates in LSDs. This observation suggests that at least some mechanisms underlying the LSD phenotype may be similar to other diseases in which defective autophagy have been observed (Lieberman et al., 2012). The study of autophagy may yet lead to the elucidation of the mechanisms of cellular death and the development of new tailored therapies for these devastating disorders (Codogno and Meijer, 2005).

3.2.4. Lipid Trafficking

SLs are essential constituents of eukaryotic cells and they are highly enriched on the PM. However, they are also internalized and transported to the LE and lysosomes where they are degraded in a stepwise fashion, culminating in the cleavage of Cer to fatty acid and sphingosine, as mentioned above (Pagano et al., 2000; Bartke and Hannun, 2009). The SL metabolic pathway displays an intricate network of reactions that is catalyzed by a specific enzyme and in some cases assisted by helper SAPs which present the substrate to the hydrolytic enzyme. This process results in the formation of a multitude of SLs, with Cer as the center of SL biosynthesis, catabolism, and as precursors of complex SLs (Pagano et al., 2000; Bartke and Hannun, 2009). Mutations in either a hydrolase or an activator protein can lead to defective hydrolysis and intracellular accumulation of lipids (Pagano et al., 2000). In addition to this primary storage, secondary storage products can also be found in LSDs (Walkley and Vanier, 2009). The most ubiquitous and best studied of these secondary storage materials are GSLs and phospholipids, as well as Chol (Walkley and Vanier, 2009).

Previous studies showed an altered lipid trafficking of SLs in some LSDs. The mechanism by which lipid trafficking is altered is not clear. However it can be related to changes in Chol levels (Vitner et al., 2010). Previous studies suggested that Chol is sequestered in the SL storage compartment owing to the fact that Chol and SLs physically associate with one another (Pagano et al., 2000; Pagano, 2003). Thus elevated Chol levels or altered Chol distribution are involved in the altered SL trafficking defect (Pagano, 2003). Fluorescent lipid analogs have been used to monitor lipid accumulation in cells from patients with LDSs (Pagano et al., 2000).

3.3. Therapy

All over the years, different therapeutic approaches were provided in patients with LSDs, to face the underlying consequences of the diseases. Infusion of plasma or plasma fractions (S Bruni, 2007), intravenous injection of exogenous enzymes extracted from human tissues (Brady, 2006), infusion of leukocytes and implantation of skin fibroblasts or amniotic cells (Yeager et al., 1985), bone marrow transplantation (BMT) (Hoogerbrugge et al., 1995), enzyme replacement therapy (Brady, 2006) and gene therapy (Dodge and Cheng, 2009) are based on this rationale and are some of the examples of therapeutic strategies that have been employed to ameliorate LSDs (S Bruni, 2007). Presently, treatment for LSDs follows two simple principles: first, the augment of the enzyme levels, genetic replacement or cellular complementation and, second, the partial inhibition of the biosynthesis rate of the parent cellular macromolecules (Cox, 2005). The substrate reduction therapy was first proposed by Radin in the 1970s (Cox, 2005) and then adapted for clinical use with particular reference to the sphingolipidoses. This treatment constitutes administration of small diffusible molecules that inhibit key biosynthetic steps in the formation of the parent substrates (Cox, 2005).

4. Farber Disease

Human ACDase (N-acylsphingosine deacylase, EC 3.5.1.23; ACDase) was originally identified by Gatt in 1963 in rat brain homogenates, but only in 1995 it was first purified from urine and characterized by Bernardo et al. (Bernardo et al., 1995). ACDase is the lysosomal enzyme catalyzing the hydrolysis of Cer to free fatty acid and sphingosine one of the final steps in SL degradation.

ACDase is a glycosylated protein consisting of two subunits, an unglycosylated α-subunit with molecular mass of 13 kDa and a *N*-glycosylated β-subunit with molecular mass of 40 kDa. These two subunits are linked by an interchain disulfide bond forming the polypeptide with molecular mass of 50 kDa (Koch et al., 1996). This heterodimeric enzyme is encoded by the *ASAH1* gene located in chromosomal region 8p21.3-p22 (Bernardo et al., 1995). Previous studies have shown the gene spans approximately 30 kb of DNA in the human genome and contains 14 exons and 13 introns.

Mutations in the ACDase gene result in FD (Farber Lipogranulomatosis, OMIM # 228000) a fatal human genetic disorder with an autosomal recessive pattern of inheritance

characterized by lysosomal accumulation of Cer in most tissues, including heart, lung, liver, and the spleen (Sugita et al., 1972; Dulaney et al., 1976; Jameson et al., 1987; Kattner et al., 1997; Ehlert et al., 2007). Deficiency of ACDase was first found in FD patients in 1972 (Park and Schuchman, 2006). However, only in 1996 was identified the first mutation in the gene encoding the ACDase in a FD patient (Koch et al., 1996). After this, several different mutations have been found in the *ASAH1* gene (Levade et al., 2009). In 2002 was constructed the first ACDase "knock-out" mouse model by Li et al.. However the lifetime was very short due to the degree of severity, (Park and Schuchman, 2006). The murine *Asah1* gene resulted in early embryonic lethality (Li et al., 2002) and the reduction of ACDase resulted in the Cer accumulation in mouse ovaries witch led to apoptosis of oocytes (Eliyahu et al., 2012), reinforcing the important role of Cer in the apoptotic pathway (Alayoubi et al., 2013). More recently, Levade and coworkers, produced the first viable model of systemic ACDase deficiency via ''knock-in'' of a known human *ASAH1* mutation [Proline (P) 362 to Arginine (R)] into a conserved murine Asah1 gene locus (P361R) (Alayoubi et al., 2013).

By electron microscopy ultrastructural abnormalities are observed, which are caused by the deficient activity of ACDase and stored Cer, including "elongated membranes", "zebra bodies", comma-shaped curvilinear tubules called Farber bodies, and spindle-shaped bodies that can be detected in fibroblasts, histiocytes, and endothelial cells (Farber et al., 1957; Schmoeckel and Hohlfed, 1979).

Diagnosis can be made by demonstrating the ACDase deficiency *in vitro* or else by showing Cer accumulation in different tissues, skin fibroblasts, white blood cells, or cultured amniocytes, together with impaired Cer turnover within living cells *in situ* (Sugita et al., 1972; Dulaney et al., 1976; Fensom et al., 1979; Bedia et al., 2010).

4.1. Clinical Phenotype

FD was first described in 1952 by Sidney Farber (Farber, 1952). To date at least 80 cases of Farber's disease have been reported in a variety of ethnic groups (Levade et al., 2009). Typical symptoms of this disease are the clustering of Cer storing histiocytes and macrophages, which form granulomas ubiquitously. Main clinical symptoms include progressive, painful arthropathy, the development of subcutaneous nodules (particularly over the dorsal surface of joints), hoarseness induced by mechanically strained cutaneous, irritability, and poor growth and development which usually begins in the first

few months and leads to death in the first two years (Amirhakimi et al., 1976; Kattner et al., 1997). The clinical diagnosis is usually made during the first year of life, and the symptoms start at this stage. Usually death occurs during the first few years (Ehlert et al., 2007).

Clinical subtypes have been distinguished based on the age of onset, mean age of death and the degree of neurologic involvement (psychomotor deterioration) (Levade et al., 1995). According to the age of onset, the severity of the symptoms and the affected tissues, Farber's disease phenotype can be divided into seven different subtypes (Table 2).

Subtypes 1 to 5 are caused by the deficient activity of ACDase, and differ with respect to severity and sites of major tissue involvement. Subtype 6 seems to be an ill-fated combination of subtype 1 of FD and Sandhoff disease (Fusch et al., 1989). And finally subtype 7, is caused by the deficiency of the sphingolipid activator protein precursor (prosaposin, pSAP) (Schnabel et al., 1992). Thus, FD can be differentiated into two groups and six subtypes according to the phenotype and clinical symptoms (Scriver, 1995; Levade et al., 1995): Group I, has unfavorable prognosis and often shows hepatic and less frequently, splenomegaly in the neonatal period. Death usually occurs within the first 14 months of life, subtype 4 belongs to this group for example. Group II with a less severe course of the disease shows less visceral involvement and only slight affection of the brain (Kattner et al., 1997). The usual onset is before the age of 1 year. Children with significant neurological involvement usually die early in infancy, whereas patients without or only mild neurological findings suffer from progressive joint deformation and contractures, subcutaneous nodules, inflammatory, periarticular granulomas, a hoarse voice and finally respiratory insufficiency caused by granuloma formation in the respiratory tract and interstitial pneumonitis leading to death in the third or fourth decade of live (Sugita et al., 1972; Ferlinz et al., 2001).

Type 1 FD affects the majority of patients (~50% of reported cases) and is the classical form of the disorder. These patients normally die in the first or second year of life (Levade et al., 2009). These patients show signs of nervous system dysfunction that include impaired psychomotor development, mild retardation and peripheral nerve involvement (Yeager et al., 2000; Ehlert et al., 2007).

Type 2 and 3, do not have so severe symptoms in CNS, when compared with type 1, however they are still severely affected with granulomatous inflammation which leads to

the formation of subcutaneous nodules, joint pain and contractures, hoarseness and respiratory insufficiency (Devi et al., 2006).

Type 4, is a very severe form with patients displaying neurological deterioration, extreme hepatosplenomegaly at birth and granulomatous infiltrations in the liver, spleen, lymphoid tissue, thymus and lungs with death in the first years of life (Antonarakis et al., 1984).

Type 5 involves progressive CNS dysfunction starting in the first 2 years of life and patients develop loss of speech, seizures, mental retardation, tetraplegia and myoclonia (Ferlinz et al., 2001).

Type 6 is a combination of FD and Sandhoff disease (an LSD caused by deficiency of hexosaminidase A and B) (Sugita et al., 1972). This patient present hoarseness, stridor (noisy breathing), scattered skin nodules, painful swelling of hand joints and ankles, and cherry-red macular spots (Sugita et al., 1972).

Type 7 FD is a single report and displays a very severe phenotype (Scriver 1995) that is not due to mutations in the *ASAH1* gene but in the pSAP-D gene whose protein products enhance the activities of lysosomal enzymes (Schnabel et al., 1992; Scriver, 1995). As a result, this patient showed combined deficiency of glucocerebrosidase, galactocerebrosidase and ceramidase (Schnabel et al., 1992).

Table 2 - Clinical phenotypes of FD patients (Taken from Levade et al., 2009).

	1 (Classical)	2 (Intermediate)	3 (Mild)	4 (Neonatal)	5 (Neurologic Progression)	6 (Comb Sandhoff)	7 (Prosaposin Deficiency)
Mean age (years)							
Onset	0.3	0.66	1.6	0	1.66	0.13	0
Death	1.45	6.25	15.8	0.16	3.6	1.1	0.3
Last follow-up	1.1	5.6	13.0	—	3.9	—	—
Nodules	100%	100%	100%	1/3	7/7	1/1	0/2
Joint involvement	100%	100%	100%	2/3	7/7	1/1	0/2
Hoarseness	100%	100%	100%	0/3	6/6	1/1	0/2
Large liver	14/20	3/7	3/7	100%	1/5	1/1	2/4
Large spleen	7/15	3/7	3/7	100%	1/5	1/1	2/4
Lung infiltrates	16/19	1/8	2/11	—	0/4	0/1	
Macular cherry-red spot	4/15	0/6	0/9	—	3/4	0/1	0/2
Lower motor neuron involvement	11/13	2/5	2/4	—	1/1	—	—
Central nervous impaired	15/22	3/10	5/9	—	7/7	1/1	2/4

4.2. Genetic basis of the disease

The first cloning of the human *ASAH1* gene was made in 1999 (Park and Schuchman, 2006). Since then, 23 different mutations have been identified in the *ASAH1* gene of Farber patients, most of them missense mutations (Table 3). These mutations are distributed along 14 exons and can affect both subunits (Bär et al., 2001; Levade et al., 2009; Muranjan et al., 2012; Alves et al., 2013).

Table 3 - Mutations in the *ASAH1* gene reported in FD patients.

DNA Change	Exon/intron	Protein Change	Subunit	Age of Death (years)	Farber Subtype	References
c.66G>C	1	Q22H	α	NI	NI	(Zhang et al., 2000)
c.67G>C	1	H23D	α	NI	NI	(Zhang et al., 2000)
c.107A>G	2	Y36C	α	2	1	(Bär et al., 2001)
c.286_288del	4	V96del	α	6.3	2	(Muramatsu et al., 2002)
C.290T>A	4	V97E	α	>10	2/3	(Muramatsu et al., 2002)
c.413A>T	6	E138V	α	30	3	(Li et al., 1999; Bär et al., 2001; Muramatsu et al., 2002)
c.412G>T	6	G128_K152del E138X	$\alpha\beta$	>1.5	3	(Bär et al., 2001)
Not found	6	G128	$\alpha\beta$	30	3	(Bär et al., 2001)
c.544C>G	8	L182V	β	0.5 brother:2	1	(Devi et al., 2006)
c.665C>A	9	T222K	β	1.9	1	(Koch et al., 1996; Li et al., 1999)
c.703G>C	9	G235R	β	>10	2/3	(Muramatsu et al., 2002)
c.760A>G	10	R254G	β	NI	NI	(Li et al., 1999; Kostik et al., 2013)
c.833C>T	11	P278L	β	>1.3	2/3	(Levade et al., 2009)
c.958A>G	12	N320D	β	2.5	6	(Zhang et al., 2000; Bär et al., 2001)
c.991G>A	12	D331N	β	>1.5	3	(Bär et al., 2001)
c.1085C>T	13	P362R	β	1.5	1	(Li et al., 1999)
IVS13+1G>T	13	N348_K366del	β	3	5	(Bär et al., 2001)
c.1187insT	14	X396L	β	NI	NI	(Zhang et al., 2000)
c.917 + 4A > G	Intron 11	p.Tyr42_Leu127delinsArgfs	β	3days	4	(Alves et al., 2013)
c.290T>G	4	p.V97G		-------	1	(Chedrawi et al., 2012)
c.502G >T	7	G168T	β	38.5 months	1	(Cvitanovic-Sojat et al., 2011)
c.1144 A > C	13	C p.K382Q)		--------	2	(Jasmi, 2012)
c.533 T > C	8	p.W185R		--------	2	(Jasmi, 2012)

As a consequence of these mutations, patients develop various symptoms, which may be more or less severe. The pathophysiological basis for the clinical heterogeneity of FD remains unknown. Presently, there is still some controversy about the correlation

between the clinical severity and either the residual ACDase enzymatic activity or the extent of Cer storage. Most of Farber patients tested so far had less than 6% of normal ACDase activity as measured in a variety of tissues (Jameson et al., 1987). Previous studies have shown that the level of stored Cer in the lysosomes is significantly correlated with the neurodegenerative course of FD and the age of death of the patient (Levade et al., 1995). However, another study reported no correlation between the levels of residual ACDase activity and the degree of Cer accumulation or symptom severity (Van Echten-Deckert et al., 1997).

4.3 Treatment

Until now there is no effective treatment for Farber's disease and most patients die at a very young age. The most frequent treatments consist on palliative treatment, administration of corticosteroids for the pain, tracheostomy to relieve respiratory difficulties, and surgery to remove the granulomas (Haraoka et al., 1997; Levade et al., 2009). Current treatments result in improvement of symptoms but they do not improve the longevity of these patients (Yeager et al., 2000). Early diagnosis is therefore important and can be done using cultured amniocytes from the amniotic fluid to assay the ACDase enzymatic activity. This method can be very useful for parents who want making selective abortion of affected fetuses (Dulaney et al., 1976).

Some treatments have focused on replacement of the missing enzyme responsible for the disease. Elizabeth Neufeld between 1960s and 1970s considered the possibility that allogeneic BMT might provide treatment and disease amelioration for LDSs (Malatack et al., 2003). These transplantations were performed in some Farber patients in whom an increase of ACDase enzymatic activity was observed. This treatment resulted in diminished joint discomfort, improved joint mobility and hoarseness. However, the prognosis was unfavorable and did not improve the longevity of these patients (Yeager et al., 2000; Zhang et al., 2000). There are also reports on improvement of the peripheral manifestations of infantile ACDase deficiency upon allogeneic BMT. However, this treatment did not result in the prevention of the progressive neurological deterioration, even when carried out in minimally symptomatic patients (Yeager et al., 2000). The economic incentive for the development of enzyme replacement therapy for FD is low, because there are few patients all over the world. However, the development of improved alternative treatment modalities remains important.

Objectives

Objectives

The aims of this thesis are:

1) Qualitative assessment of primary and secondary buildup of lipids in Farber disease patients' derived fibroblasts.

2) Characterization of the C16-Cer/Chol microdomains in Farber disease and other LSDs patients' derived fibroblasts.

Materials and Methods

Materials and Methods

1. Materials

1.1 Cell Lines

All cell lines from human skin fibroblasts, 20015, 20016, 20017, 20018, 2314, 2315, 5752, 18313 (see table 4) and 2316 (Farber carrier), were obtained from NIGMS Human Genetic Cell Repository. The patient's cultured fibroblasts, Moh mock inf 5x, Farber transduced fibroblasts with ACDase cDNA, Moh corrected 5x and x10 retroviral correction (Accer 5x and 10x), (Medin et al., 1999) and Schr (prosaposin deficiency) (Sap) (Chatelut et al., 1997), were a kind gift from Professor Thierry Levade (Laboratoire de Biochimie, Maladies Métaboliques, Institut Louis Bugnard, Toulouse, France). Normal human skin fibroblasts (Wild Type 1 to 4) (WT) and fibroblasts from Niemann-Pick type B (NPB), Niemann-Pick type C1 (NP-C1), Gaucher type 2, Krabbe, Fabry disease, mucopolysaccharidoses **(**MLD), GM1-gangliosidosis (GM1), neuronal ceroid lipofuscinoses type 3 (CLN-3) and I-cell disease (ICD) were kindly provided by Doctor Lúcia Lacerda from the National Health Institute Dr. Ricardo Jorge Centro de Genética Médica Dr. Jacinto de Magalhães, Portugal.

Table **4** - NIGMS cell lines from Farber patients, specifying age of death and type of Farber disease, according to the classification of Moser

Farber patients	Age of death	Type	Rated
20015	2 years	Type 1	Classical
20016	2.5 years	Type 6	Combiation Sandhoff
20017	3 years	Type 5	Neurologic Progression
20018	22 months	Type 1	Classical
2314	6 years	Type 2-3	Mild
2315	30 years	Type 3	Mild
5752	6 months	Type 4	Neonatal
18313	11 months	Type 4	Neonatal

1.2 Cell culture

Cells were cultured in Dulbecco's Modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), both from Gibco Invitrogen (Carlsbad, CA), and penicillin (100 U/ml), and streptomycin (100 µg/ml) (BD Biosciences, San Jose, CA).

1.3 Antibodies

Mouse monoclonal antibody anti-GM2 (IgM) was kindly provided by Doctor Tai (Department of Neurobiology, School of Life Sciences, Tottori University Faculty of Medicine, Yonago, Japan); anti-mouse $(Fab')_2$ IgM µ-chain specific, conjugated with fluorescein was obtained from Sigma Aldrich (St Louis, Missouri, MO). The following antibodies were used: anti-voltage-dependent anion-selective channel protein 1 (VDAC1), anti-LAMP2a, anti-calnexin and anti-Rab7 (Abcam, Cambridge, UK); anti-Rab5 (Santa

Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-Golga5 (Sigma-Aldrich, St Louis, Missouri, MO); Cy2- and Cy3-labeled secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA). An anti-mouse Fab fragment conjugated to 1.2 nm colloidal gold was from Nanoprobes (Yaphank, NY, USA).

1.4 Lipids and Lipid Derivatives

BODIPY-lactosylceramide (BODIPY-LacCer) was kindly provided by Dr. Robert Bittman (Department of Chemistry and Biochemistry, Queens College of The City University of New York), BODIPY-C5-Cer (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl) and BODIPY-C5-SM (N-[5-(5,7-dimethyl boron dipyrromethene difluoride)-1-pentanoyl]-D-*erythro*-sphingomyelin) were purchased from Molecular probes, Filipin complex was obtained from Sigma Aldrich (St Louis, Missouri, MO).

1.5 Other Reagents

Silica gel 60 thin-layer chromatography (TLC) 20x20 plates were from Merck (Darmstadt, Germany), bovine serum albumin (BSA) from Sigma Aldrich St Louis (Missouri, MO), protease inhibitor cocktail (Roche Diagnostics Ltd, Mannheim, Germany), glass coverslips (Fisher Scientific), elvanol mounting medium (Molecular Probes-Invitrogen), formaldehyde solution from Sigma Aldrich St Louis (Missouri, MO), vectorShield was obtained from (Vector Laboratories), ethylenediamine tetraacetic acid (EDTA) phenol red solution from Gibco Invitrogen (Carlsbad, CA) and Lipoprotein-deficient Serum (LPDS) from Sigma (Missouri, MO). 2-hydroxypropyl-β-cyclodextrin (CD) was from Sigma-Aldrich (St Louis, Missouri, MO). Wheat germ agglutinin (WGA), Texas Red and 4'6-diamidino-2-phenylindole (DAPI) were from Molecular Probes (Eugene, OR, USA). All other reagents or solvents were of the highest grade.

2. Methods

2.1. Cell culture

Fibroblasts were maintained in culture medium DMEM supplemented with 10% of FBS and antibiotics (10.000 U/ml streptomycin / penicillin and 1 mg/ml fungizone), at 37ºC in 5% CO_2. A 20mmol/l Tris/0.25mol/l sucrose solution, pH 7.4, was used as washing solution. Cell passage was achieved by flowing trypsin/EDTA phenol red solution.

2.2. Lipid Extractions and High performance thin layer chromatography (HPTLC) Analysis

Cell pellets were suspended in 1mL of methanol and sonicated twice (15 seconds each time) (W-375 Ultra-sonic model, Heat Ultrasonics-Systems, Inc.). Lipids were extracted and separated by reversed-phase chromatography according to the procedure previously described (Bligh and Dyer, 1959). Briefly, after lipid extraction, Bond Elut C18 columns were placed in a Vac Elut column holder (Varian, USA). After column washing, the mixture was loaded and two fractions recovered as follows: the acid lipids were eluted with methanol/water (12:1 v/v) and neutral lipids with chloroform/methanol (1:2 v/v). The samples were dried under nitrogen stream and reconstituted with chloroform/methanol (1:2 v/v). Lipids were resolved by HPTLC (Linomat 5, Camag, Switzerland) developed in chloroform/methanol/ammonia (95:5:0.8 v/v/v) and visualized by incubating the plate at 120ºC for 15 minutes after spraying with 1% solution of anisaldehyde in 80% of sulphuric acid after the plate was scanned.

2.3. Acid Ceramidase Assay

Cell pellets were homogenate and added to a final volume of 200µL sucrose-EDTA buffer (0,25M sucrose, 0,001M EDTA) containing a protease inhibitor cocktail diluted 1:200. Then citric acid was added in final concentration 0.1M and pH=4. The reaction initiated by addition of 4nmol of C_{12}-NBD-Cer. The reaction proceeded for 3 hours at 37ºC in the dark and was terminated by addition of three volumes of chloroform:methanol (1:2 by volume). Lipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959), and lipids from the lower phase were separated by TLC using chloroform: methanol: 9.8mM $CaCl_2$ (60:35:8 by volume) as the developing solvent. NBD-labeled SLs were

identified using authentic standards by the Fluor-S Max device and the bands were quantified by using Image Quant program (Amersham Biosciences).

2.4. Cell Staining

2.4.1. Incubation with lipids and lipid derivatives

For BODIPY-C5-Cer cell incubation assay, the procedure was performed as described (Pagano et al., 1991). HSF cultures were plated in glass cover slips and incubated in LPDS media at 37ºC for 4 days, followed by 24h incubation in normal medium containing 100 µg/mL of LDL by incubating cultures with 2µM of lipid analogue for 30 min at 37ºC. For BODIPY-LacCer assay, HSF cultures were plated in glass cover slips and incubated in LPDS media at 37ºC for 4 days, followed by 24h incubation in normal medium containing 100 µg/mL of LDL. The assay was then carried out as previously described (Martin and Pagano, 1994) using the 5µM of BODIPY-LacCer and an incubation period of 45 min. For BODIPY-C_5-SM cell assay, the fibroblasts were plated in glass cover slips and incubated in LPDS media at 37ºC for 4 days, followed by 24h incubation in normal medium containing 100 µg/mL of LDL and then labeled with 2µM BODIPY-C5-SM complexed with BSA for 30 min at 37°C. The cells were imaged with a Nikon Eclipse E400 fluorescence microscope (λ_{exc} = 494 nm; λ_{em} = 518 nm) and charge-coupled device Nikon Coolpix 950 camera. At least two independent experiments were performed.

2.4.2. Immunofluorescence staining of GM2

Cells were fixed with 3.7% formaldehyde in PBS pH 7.4 for 10 min at RT, rinsed twice with PBS, and permeabilized with 0.5% Triton X-100/PBS for 10 min at RT. After two washes, cells were blocked with PBS containing 1% BSA for 30 min. Cells were then incubated with monoclonal primary antibody anti-GM2 (IgM) (Kotani et al., 1992) previously diluted 1:50 in PBS, for 12–16 h at 4 °C. After washing three times with PBS cells were incubated for 1 h with secondary antibody (anti-mouse $(Fab')_2$ IgM µ-chain specific, conjugated with fluorescein) previously diluted 1:75 (Sigma). After washing with PBS, coverslips were mounted with VectorShield (Vector Laboratories), imaged with a Nikon Eclipse E400 fluorescence microscope (λ_{exc} = 494 nm; λ_{em} = 518 nm) and charge-coupled device Nikon Coolpix 950 camera.

2.4.3. Filipin staining

Fibroblasts were stained with filipin and viewed by fluorescence microscopy according to Kruth et al. (Kruth et al., 1986). Briefly, cells were plated into chamber slides (Labteck slip, Nunc) in DMEM supplemented with 10% of FBS, at 37 °C with 5% of CO_2 for 24 h. The media was replaced by DMEM with 10% LPDS and cells cultured for 3 days before human LDL was added (100 µg/ml). The LDL fraction was prepared using blood from normal donors following the procedure described by Havel et al. (Havel et al., 1955). After a 24-h incubation with LDL in 10% LPDS media, cells were washed with PBS (three times for 5 min), fixed with formaldehyde 3.7% (v/v) for 10 min at room temperature. After washing twice with PBS, 0.1 mg/ml of fresh filipin was added to each slide and kept in the dark for 1 h. After washing in PBS, slides were mounted using VectaShield and visualized with a Nikon Eclipse E400 fluorescence microscope (λ_{exc} = 364 nm; λ_{em} = 475 nm).

2.5. In *vitro* Acid Sphingomyelinase Enzymatic Assay

The enzymatic activity of intracellular ASMase was determined essentially as previously described (Van Diggelen et al., 2005). Briefly, cell pellets were extracted in water by sonication (W-375 Ultra-sonic model, Heat Ultrasonics-Systems, Inc.). After centrifugation for 30 min at 15 000 *g* the supernatant, referred to as extract, was assayed, in duplicate, using Bicinchoninic acid (BCA) to determine protein concentration. In cell extracts, the enzymatic activity of ASMase was determined against the fluorogenic synthetic substrate 6-hexadecanoylamino-4-methylumbelliferylphosphorylcholine (HMU-PC). The fluorescence was measured on a Hitachi F2000 spectrofluorometer. Standard solutions of 4-methylumbelliferone were used to calculate specific enzymatic activity. The activity of another lysosomal enzyme, β-galactoside, was also assessed as reference enzyme.

2.6. Cell culture incubation with U18666A or desipramine

Cells were plated in 3mL of culture medium on 60 mm dishes. The next day, the medium was removed and replaced with fresh medium supplemented with 10% LPDS.

After three days, the medium was renewed and supplemented with 4.5 µg/mL U18666A for 24 hours or 3µM desipramine for 1 hour. Cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min and stained with 50 µg/mL of freshly prepared filipin solution in PBS for 1 hour at room temperature and stored in the dark. For *in vitro* acid sphingomyelinase enzymatic activity assay, cells were grown in 75-cm^2 tissue culture flasks, harvested and immediately used or stored at -20°C.

2.7. Studies with Anti C16-Cer/Chol antibody

2.7.1. Production, screening and purification

The Antibody anti C16- or C18-ceramide (anti-Cer/Chol) antibody was produced as previously described (Scheffer et al., 2006).

The antibodies were initially purified from ascites fluid by affinity chromatography using an immunoPure IgM purification Kit (Thermo scientific-Pierce) according to manufacturer´s instructions. Only fractions with optical densities of >0.2 were pooled. The purified antibody was extensively dialyzed against PBS and stored at 4ºC. Antibody concentrations were calculated using the antibody absorbance spectrum at 200-400nm. The antibody is stable up to 7 days after purification.

2.7.2. Cell labeling

Cells were cultured on 12-13 mm glass coverslips to a confluency of ~70%. Cells were fixed by incubating with 3% paraformaldehyde in PBS at room temperature for 45 min, rinsed three times with PBS containing 0,003% BSA, first wash for 1 hour the following for 5 minutes each. After incubated for 1 hour with anti-C16-Cer/Chol antibody (10 µg/ml), cultures were rinsed three times with PBS containing 0,003% BSA 5 min each, and then incubated 45 min with the appropriate secondary antibody. Cells were mounted in Elvanol (12% elvanol, 3% glycerol, 60 mm Tris, pH 8.5, 1 mg/ml p-phenylenediamine) and observed 24 hours later. Acquisition was performed using a confocal FV1000 on Olympus IX81 microscope, using 1.35 NA UPLSAPO 60x oil objective (λ_{exc} = 490 nm; λ_{em} = 525 nm).

2.7.3. Immunofluorescent localization of C16-Cer/Chol domains

Cells were cultured on 12-13 mm glass coverslips to a confluency of ~70%. Cells were fixed by incubating with 3% paraformaldehyde in PBS at room temperature for 50 min, rinsed three times with PBS containing 0,003% BSA, first wash for 1 hour the following for 5 minutes each. After incubated for 1 hour with anti-C16/Chol antibody (10 µg/ml), alone and with organelle markers. Antibodies to Rab 7 for endosomes, were used at a dilution of 1:50, Rab 5, for early endosomes, at 1:50, Golga5 for the Golgi, at 1:100, voltage-dependent anion-selective channel protein 1 (VDAC1) for mitochondria, at 1:180, Calnexin for ER, at 1:250 and Lamp 1 for lysosomes, at 1:180. Cultures were rinsed three times with PBS containing 0,003% BSA 5 min each, and then incubated 45 min with the appropriate secondary antibodies. The nucleus was stained using DAPI at a dilution 5:5000 for 5 min. Cells were mounted in Elvanol and observed 24 hours later. Time-lapse acquisitions were performed using an Olympus confocal microscope.

2.7.4. Incubation with 2-hydroxypropyl-β-cyclodextrin (CD)

Cells were cultured on 12-13 mm glass coverslips to a confluency of ~70%.
Fibroblasts were treated with 100 µM CD for 48h.

2.8. Electron microscopy

Fibroblasts of WT and of Farber (5752) cells grown on tissue culture plates were gently removed by washing. Fixation and labeling were performed as explained for fluorescence microscopy with the exception of the gold-conjugated (1.2 nm) secondary antibodies that were used. Enhancement of these particles, to a size of 10–30 nm, was performed using a Gold Enhancement kit (Nanoprobes, Yaphank, NY). The pellet was left overnight in 2% glutaraldehyde to allow further fixation. Prior to freezing, the pellets were washed by centrifugation (2900g, 4 min). Transmission electron microscopy (TEM) was performed using a chemically fixed cell pellet, which was labeled and enhanced using the same methods above mentioned. For immunolabeling the Tokuyasu preparation method

was used (Tokuyasu, 1973). Briefly, samples were infiltrated, cooled and embedded in gelatin and fixed with 2% glutaraldehyde on ice, cryo-protected in sucrose and vitrified by plunging in liquid N2. The frozen samples were cryo-sectioned using a Leica EM FC6 cryomicrotome, and then transferred to bare 200-mesh nickel or format-coated TEM grids. Sucrose was removed; grids were stained with uranyl acetate, and embedded in methyl cellulose. Samples were viewed on a TEM, T12-Technai TEM microscope operating at 120 kV. Images were recorded on an Eagle 2 K_ 2 K FEI camera (Eindhoven, Netherlands)

Results

Results

1. Rationale for the selection of FD and control cell lines

Fibroblasts from FD are characterized by a primary accumulation of Cer. It is now known that in several LSDs secondary lipid storage also occurs, as in NP-C1, mucopolysaccharidoses, as well as some glycoproteinoses and ceroid lipofuscinoses, where GM2 and GM3 gangliosides levels are elevated as a consequence of the primary accumulation of Chol, glycosaminoglycans (GAG), oligosaccharides and ceroid lipofuscin-like autofluorescent lipopigments respectively. Secondary lipid accumulation in cells from FD patients has, so far, not been addressed. Therefore, one of the major aims of the present work is to investigate if there are secondary lipid accumulations in fibroblasts from FD patients and their implications in lipid trafficking. To this end, cells from the two most common forms of FD, subtypes 1 and 3, and the most severe form of FD described to date (caused by *ASAH1* gene defects), subtype 4, were studied. The results indicated that, in addition to Cer, these cells also accumulated Chol (as will be described below in detail) and this prompted performing studies using an antibody that specifically recognizes Chol and C18/C16-Cer structured domains (Medin et al., 1999; Goldschmidt-Arzi et al., 2011). The underlying aim of this part of the study was to identify if these lipids co-accumulated in the same cellular regions and thus potentiating the formation of these Cer/Chol-enriched structured domains. In these studies, in addition to the cells described above, fibroblasts from FD patients from all Farber types, 1 to 7 (see table 4) were also included in order to make the study more complete and representative, and to evaluate for a potential correlation between the severity of the mutation and the formation of Cer/Chol domains. To investigate if these domains were specific of FD or a common feature to other LSDs, these studies were also performed in a cell line representative of specific LSD. Accordingly, fibroblasts from NP-C1, NPB, Gaucher type 2, Krabbe disease, Fabry disease, metachromatic leukodystrophy, GM1 gangliosidosis, CLN-3 disease and I-Cell disease were used (Table 1). As described in the Introduction, each of these diseases accumulates a different substrate of an impaired enzyme: in NP-C1 the mutation causes a deficiency in Chol transporter, hence Chol accumulates. NPB disease is caused by the deficiency of ASMase, hence SM is accumulated; Gaucher type 2 disease is caused by glucocerebrosidase deficiency and hence glucosylceramide accumulation occurs; Krabbe disease is caused by a deficiency in galactocerebrosidase and as a result

galactosylceramide accumulates; Fabry disease is caused by the deficiency of alpha galactosidase A and accumulation of globotriaosylceramide (Gb3) ensues; metachromatic leukodystrophy (also called Arylsulfatase) is a disease caused by the deficiency of arylsulfatase A and without this enzyme, sulfatides accumulate in many tissues; GM1 is caused by the deficiency of beta-galactosidase and consequent accumulation of GM1 ganglioside. CLN-3 disease is the juvenile form of Batten disease where there is an accumulation of lipofuscins. ICD is an inclusion cell disease, in which the phosphotransferase labeling a M6P on proteins designated to the lysosome is deficient; hence all proteins end up in inclusion bodies and later on out of the cell. In this study were used four different WT cell lines.

2. Characterization of FD and control cell lines

Prior to investigate downstream effects of lysosomal Cer storage in ACDase-deficient cells, the level of total Cer was first assessed by lipid extraction and fractionation, and analysis by HTPLC (Figure 8). As could be anticipated, the level of total Cer was higher in FD cell lines compared to normal control cells. Overexpression of ACDase corrected this alteration, in agreement with previous studies (Medin et al., 1999; Elojeimy et al., 2006).

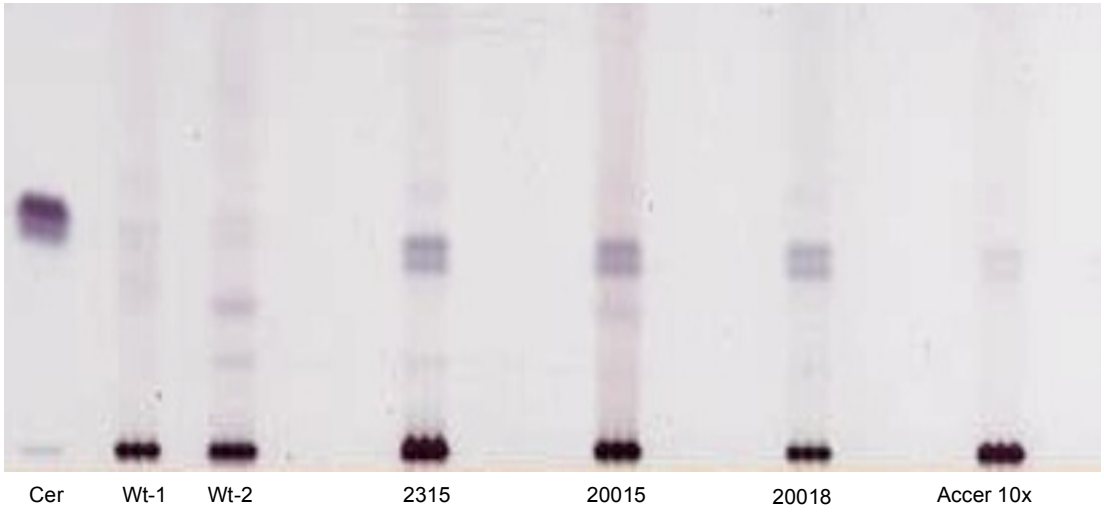

Figure 8 - Qualitative assessment of intracellular Cer levels in cultured human skin fibroblasts (HSF).

Cells were harvested, subjected to lipid extraction and fractionation and analyzed by HTPLC as described in Materials and Methods. Cer levels in normal control cells (Wt-1 and Wt-2) were compared to three different Farber cell lines (2315 (Type 3); 20015 (Type 1); 20018 (Type 1)) and to FD cells overexpressing ACDase (retro viral correction (Accer 10x)).

The band doublet represents ceramides with fatty acids of distinct length (C16-C18 and C20-C24). Cer-20 was used as standard.

To further characterize these cells, the activity of ACDase was quantified in FD, WT and FD carrier fibroblasts. To this end, C12-NBD-Cer was added as a substrate for the reaction. The lipids were separate by TLC using chloroform: methanol: 9.8 mM CaCl2 (60:35:8 by volume) and the bands corresponding to the fluorescent acyl chain degradation product were quantified using the Image Quant program (Amersham Biosciences) (Figure 9). The activity of ACDase was much lower in all Farber patients' fibroblasts, as can be seen by the very low substrate conversion compared to WT fibroblasts. The activity of the enzyme was also reduced in Farber carrier cells although to a much lower extent compared to WT (≈70%). Within the different FD subtypes there are some variations in enzyme activity, where the most severe cases, which belong to subtype 4 and 7, seem to have the lowest enzyme activity (≈4% and ≈7%, respectively, of substrate conversion). In all other FD cells the % of Cer conversion is slightly higher, between ≈ 8-15%, further confirming the correlation between the severity of the disease and the activity of ACDase, as previously shown (Medin et al., 1999). ACDase activity was also determined in an immortalized Farber patient cell line (Moh Mock inf 5x) and its retroviral corrected version (Accer 5x and x10) (Figure 9). As previously shown (Medin et al., 1999) Moh Mock inf 5x was classified as subtype 4, and the activity of ACDase was reported to be very low in these cells (Medin et al., 1999). This is further confirmed in the present study, where the activity of the enzyme is comparable to other FD cells from subtype 4, i.e., ≈4% of Cer conversion. The activity of the enzyme was reestablished in the corrected cell line (Accer 5x and 10x) and a very high conversion was obtained, which corresponded to almost two times the WT percent conversion.

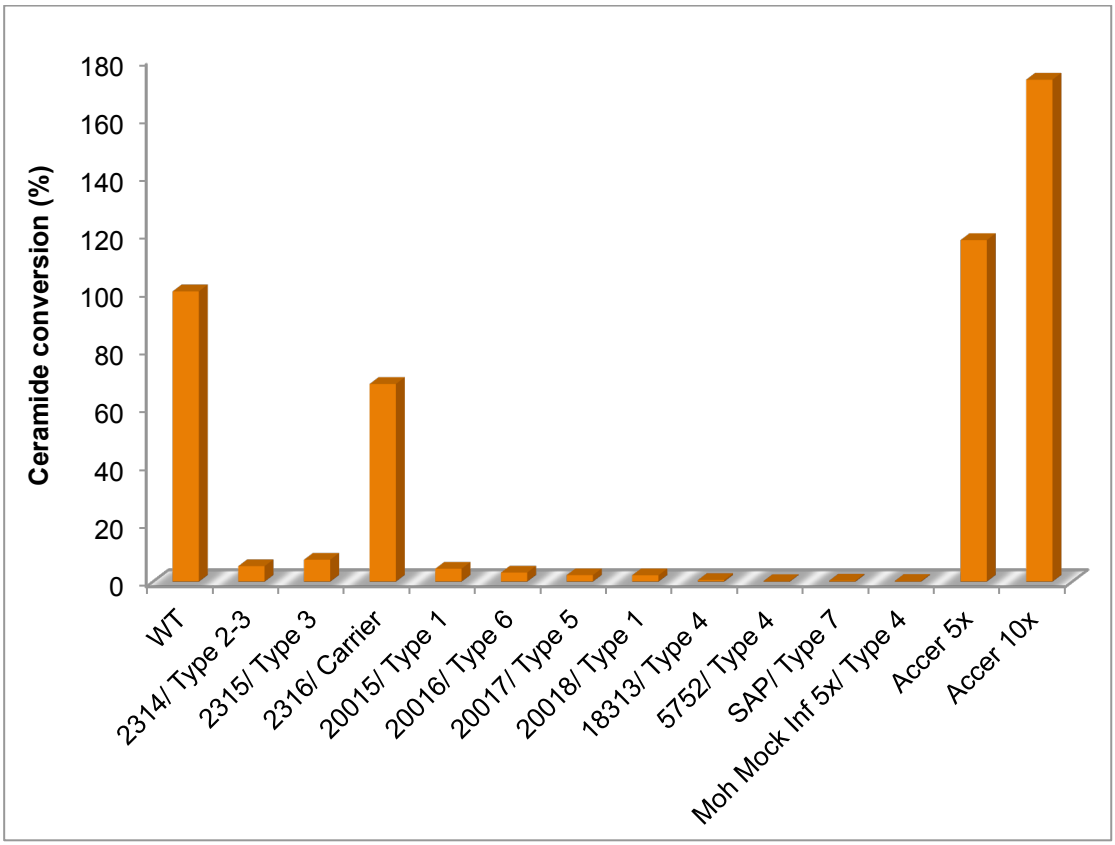

Figure 9 - In vitro activity assay of ACDase in HSF.

Percent of ACDase substrate conversion quantified in WT, Farber carrier (2316), Farber fibroblasts (2314; 2315; 20015; 20016; 20017; 20018; 18313; 5752; Sap and Moh Mock inf 5x) and retroviral corrected Farber fibroblasts (Accer 5x and x10) The lipids were extracted by the method described by Bligh and Dyer, 1959. C12-NBD-Cer was used as a substrate for the reaction and the acyl chain degradation product was measured. Bands were quantified by using Image Quant program (Amersham Biosciences).

3. Secondary Lipid Accumulation in FD

Previous studies showed that fluorescent lipid analogs can be useful tools to study lipid traffic between the Golgi apparatus and the PM of living cells (Pagano, 2003). Furthermore, these studies highlight the potential of this assay to be used as an initial general screen for a broad group of lipid-storage diseases (Pagano et al., 2000; Pagano, 2003).

The present study took advantage of these fluorescent lipid analogs to study lipid trafficking in FD cells. BODIPY-C5-Cer was used to study lipid traffic at the Golgi

complex and BODIPY-LacCer to study lipid transport along the endocytic pathway of HSF.

Lipid analogs labeled with the BODIPY fluorophore were selected because BODIPY has unique spectral properties when compared to other fluorophores such as pyrene or NBD (Pagano et al., 1991; 2000; Marks et al., 2008). BODIPY is more photostable and the fluorescence emission is relatively insensitive to environmental factors like medium polarity, pH and oxygen (Pagano, 2003; Marks et al., 2008). In addition, it is possible to estimate the concentration of BODIPY-labeled lipid analogs, because the fluorescence properties of the probe change with its local concentration (Pagano et al., 1991; 2000). This is because two molecules of the dimethyl BODIPY fluorophore can form an excited state dimer, termed an excimer, that exhibits a second emission peak red-shifted from that arising from the excited monomer (Pagano et al., 1991; 2000; Marks et al., 2008). Accordingly, when BODIPY fluorophore is present in membranes at relatively high concentrations, the typical green emission of the monomer is partially quenched and a second emission peak, derived from excimer emission, is observed at higher (red) wavelengths (620 nm). Due to these special properties, BODIPY can be used to monitor lipid accumulation in the lysosomes of SL storage diseases. In the present study, the intracellular lipid distribution in fibroblasts from patients with various SL-storage diseases such as Farber, NPC, and NPB was studied. To this end, fluorescence imaging of cells labeled with BODIPY-lipid analogs was performed using 450–490 nm for excitation, and the emission was collected in three different channels: green (520–560 nm), green+red (520 nm) and red (590 nm) (Puri et al., 1999; Marks et al., 2008).

3.1. Trafficking of endocytosed Cer

The distribution of endocytosed Cer was assessed in different cell types such as: ACDase-deficient (2315 (Type 3); 20015 (Type 1); 20018 (Type 1); Moh Mock inf 5x (Type 4)), FD cells overexpressing ACDase (Accer 10x) and WT. The cells were treated with the analogue BODIPY-C5-Cer and analyzed by fluorescence microscopy (Figure 10).

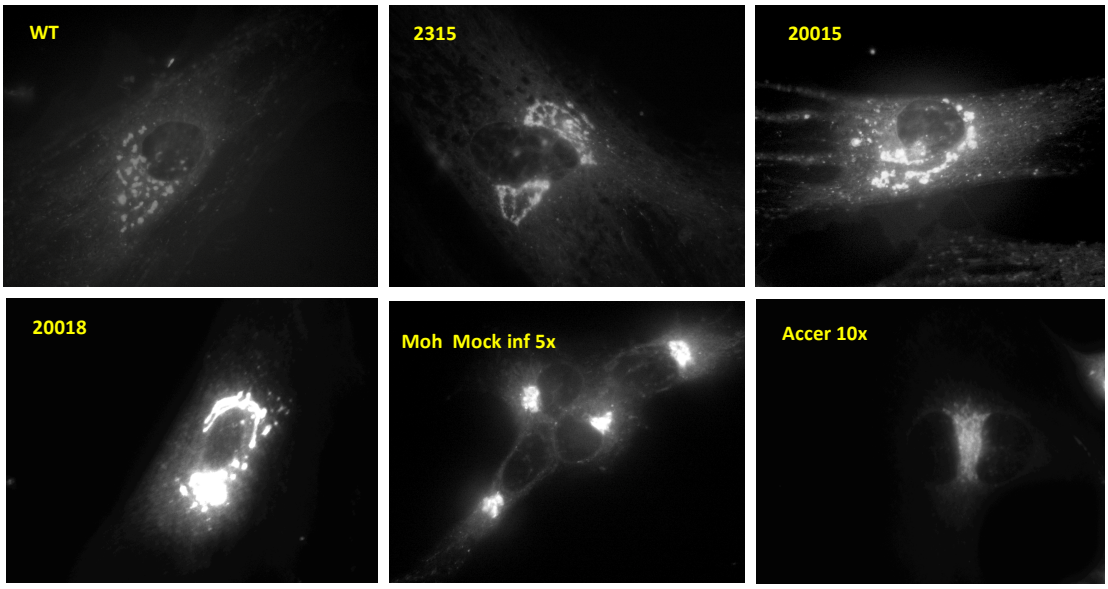

Figure 10 - Endocytosis and intracellular distribution of BODIPY-C5-Cer in HSF.

HSF were plated in glass cover slips and incubated in LPDS media at 37ºC for 4 days, followed by 24h incubation in normal medium containing 100µg/mL of LDL. Then the cells were incubated for 45 min with the lipid analog at 37°C in culture medium containing 2µM BODIPY-C5-Cer/BSA, washed, and further incubated for 1 h at 37°C in culture medium (Pagano et al., 1991; 2000a; Castro et al., 2009). The fluorescent lipid present at the PM was removed by back-exchange with 5% BSA and analyzed by fluorescence microscopy. WT; FD cells (2315 (Type 3); 20015 (Type 1); 20018 (Type 1); Moh Mock inf 5x) and cells overexpressing ACDase (Accer 10x) were treated with the analogue BODIPY-C5-Cer and imaged with a Nikon Eclipse E400 fluorescence microscope ($\lambda_{exc.}$ = 494 nm; $\lambda_{emi.}$ = 518 nm) and charge-coupled device Nikon Coolpix 950 camera. Representative images from n=30 cells, are shown.

Imaging of the cells by fluorescence microscopy using the green+red channel, revealed a bright perinuclear and reticular pattern of fluorescence coincident to the Golgi complex. However, the fluorescence intensity was considerably higher in FD cells (20018; Moh Mock inf 5x) and a shift from green to red wavelengths, indicative of BODIPY excimer formation and thus, of a higher local concentration of probe, most probably in Golgi apparatus membranes, was noticed in FD fibroblasts (20018; Moh Mock inf 5x). These results suggest that BODIPY-C5-Cer and its metabolites (SM and glucosylceramide) can accumulate in the Golgi apparatus (Puri et al., 1999; Marks et al., 2008). Overexpression of ACDase reduced the Golgi Cer levels seen in the disease cell counterparts. Due to technical limitations it was impossible to make a quantitative comparison of the fluorescence intensities and show the different channel colours.

3.2. Trafficking of endocytosed LacCer

The distribution of the internalized fluorescent glycosphingolipid lactosylceramide-analog was assessed using BODIPY-LacCer. In this study, in addition to the cell lines described above, NPC fibroblasts, which accumulate cholesterol and BODIPY-LacCer (Puri et al., 1999; 2001), were used as a positive control. Internalization of BODIPY-LacCer results in a punctuate endosomal pattern of fluorescence and enhanced Golgi labeling of cells from Farber patients (20015 (Type 1); 20018 (Type 1) and Moh Mock inf 5x (Type 4)) and NPC. In contrast, control cell line, corrected Farber Cell line Accer 10x and Farber cell line 2315 (Type 3) the fluorescent LacCer was uniquely targeted to the Golgi (Figure 11). Farber cell line 2315 corresponds to a mild form of the disease and therefore, it was antecipated that alterations in endocytic trafficking subsequent of the disease were not as marked as observed for the other FD cells. These results are in agreement with previous reports for this cell line (Pagano et al., 2000).

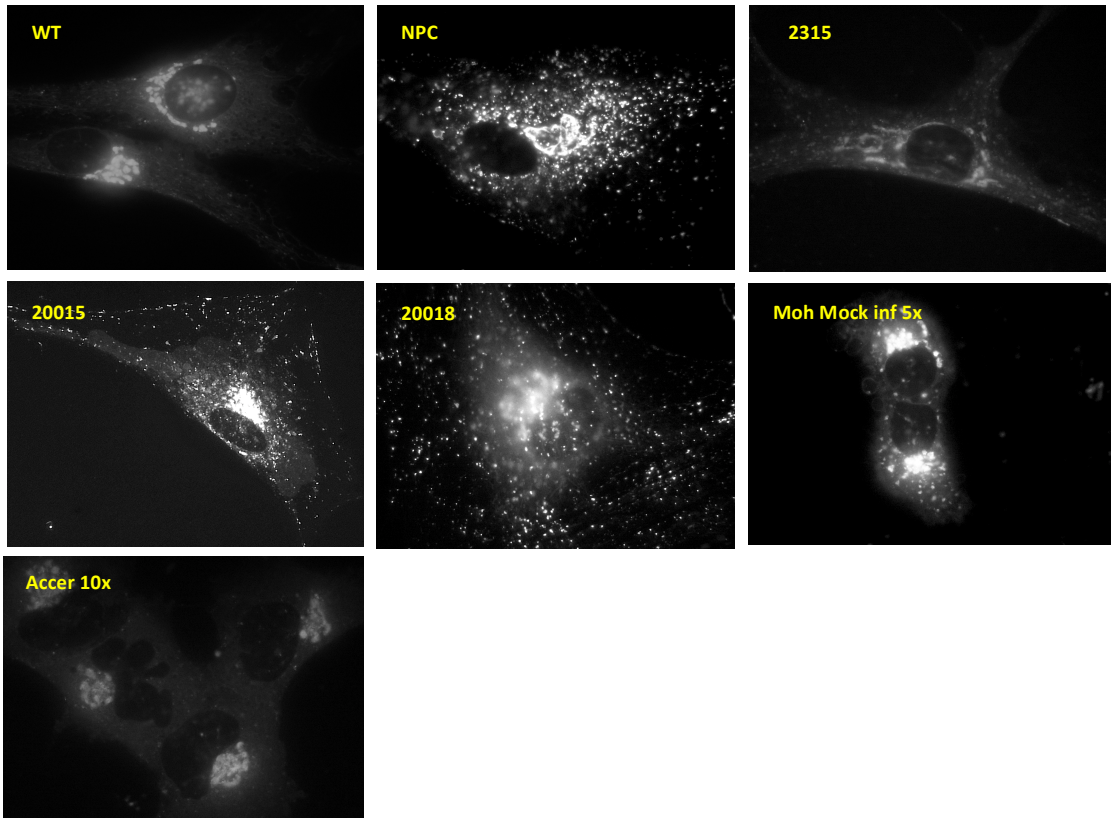

Figure 11 - Endocytosis and intracellular distribution of BODIPY-LacCer.

Briefly HSF from normal cells (WT), Farber patients (2315; 20015; 20018; Moh Mock inf 5x) and NPC patients were plated in glass cover slips and incubated in LPDS media at 37ºC for 4 days, followed by a 24h incubation in normal medium containing 100µg/mL of LDL; labeled for 45 min at 37ºC with 5µM BODIPY-LacCer, washed and then chased for 1 h at 37°C. Fluorescent lipid present at the PM was removed by back-exchange with 5% BSA and imaged with a Nikon Eclipse E400 fluorescence microscope (λexc. = 494 nm; λemi. = 518 nm) and charge-coupled device Nikon Coolpix 950 camera. Representative images from n=30 cells are shown.

As observed for cells labeled with the fluorescent BODIPY-C5-Cer, the fluorescence of BODIPY-LacCer displays a red-shift in its emission in fibroblasts from FD cell lines 20015, 20018 and Moh Mock inf 5x, as well as in the positive control NPC cells. These results suggest an increased probe concentration in Golgi apparatus. This was not observed for the other studied cells.

BODIPY-LacCer is internalized from the PM to the Golgi complex in normal human skin fibroblasts, but is targeted predominantly to endosomes and lysosomes in fibroblasts from LSDs, suggesting a common mechanism of cellular dysfunction in these biochemically distinct disorders (Chen et al., 1997; Puri et al., 1999;2001; Pagano et al., 2000; Marks et al., 2008).

For the same reason as mentioned above it was not possible to quantify the intensity of the probe and to present the different channels in colored images.

3.3 Trafficking of endocytosed SM

Previous studies showed the fluorescent lipid BODIPY-C_5-SM is transported predominantly to the Golgi apparatus, while in the mutant NPB cells, which have a defect in the lysosomal degradation of SM, the fluorescent lipid is transported to the lysosomes and accumulates there (Koval and Pagano, 1990). After adding BODIPY-C_5-SM to these cells, the concentration of the lipid analog increases in the lysosomes and there is a shift in its fluorescence emission from green to red wavelengths (Pagano et al., 2000a; Marks et al., 2008). In order to evaluate for putative alterations in SM trafficking in FD, different cell lines FD 2315 (Type 3); 20015 (Type 1); 20018 (Type 1); Moh Mock inf 5x (Type 4) were labeled with BODIPY-C5-SM. These studies were also performed in cells overexpressing ACDase (Accer 10x), WT cells, and NPB and NPC cells were used as a positive control. Representative results of BODIPY-C5-SM distribution in these cells lines are shown in figure 12.

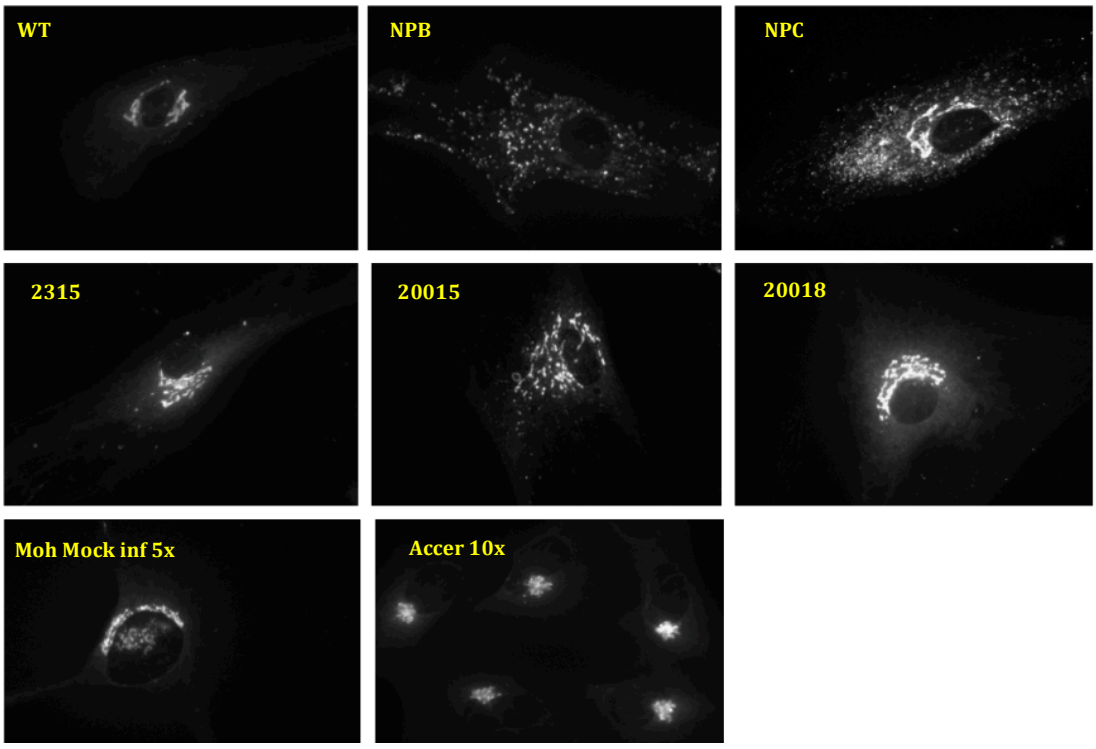

Figure 12 - Endocytosis and intracellular distribution of BODIPY-C5-SM.

Normal (WT), FD (2315; 20015; 20018; Moh Mock inf 5x), NPB, NPC and cells overexpressing AC (Accer 10x) HSF after incubation with BODIPY-C_5-SM. Cells were plated in glass cover slips and incubated in LPDS media at 37ºC for 4 days, followed by a 24h incubation in normal medium containing 100µg/mL of LDL. The cells were then labeled with 2µM BODIPY-C_5- SM complex with BSA for 30 min at 37°C, washed, and chased for 1 h at 37°C before back-exchange (Chen et al., 1997). Cells were imaged with a Nikon Eclipse E400 fluorescence microscope (λexc. = 494 nm; λemi. = 518 nm) and charge-coupled device Nikon Coolpix 950 camera. Representative images from n=30 cells, are shown.

Some important differences in the labeling of the normal versus NPB fibroblasts are observed, which are in agreement with previous literature reports (Walkley and Vanier, 2009). First, in normal cells, most of the fluorescence is seen at the Golgi apparatus, (Pagano, 1990; Pagano et al., 1991; Rosenwald and Pagano, 1993), presented a pattern reticular and perinuclear, which is brightly labeled in the green and red microscope channels. In NPB cells, a bright punctate pattern of fluorescence is observed in both the 'green' and the 'red' microscope channels. This fluorescence pattern can also be observed in NPC fibroblasts. In Farber cells and cells overexpressing AC the fluorescence pattern was reticular and perinuclear, similar to observed in normal control cells. These results suggest that trafficking of endocytosed SM is largely unaffected in cells from FD patients.

3.4. Cell ganglioside GM2 imbalance by staining with antibody anti-GM2

Secondary GSL accumulation has been documented in HSF (Walkley and Vanier, 2009) and mouse models in a wide range of LSDs, with or without primary defects in ganglioside degradation (McGlynn et al., 2004). This accumulation is accompanied by sequestration of free Chol in a manner similar to that observed in primary gangliosidoses (Walkley and Vanier, 2009). To evaluate whether gangliosides accumulation also take place in FD, we investigated the accumulation of ganglioside GM2 in ACDase-deficient fibroblasts by immunofluorescence microscopy (Figure 13).

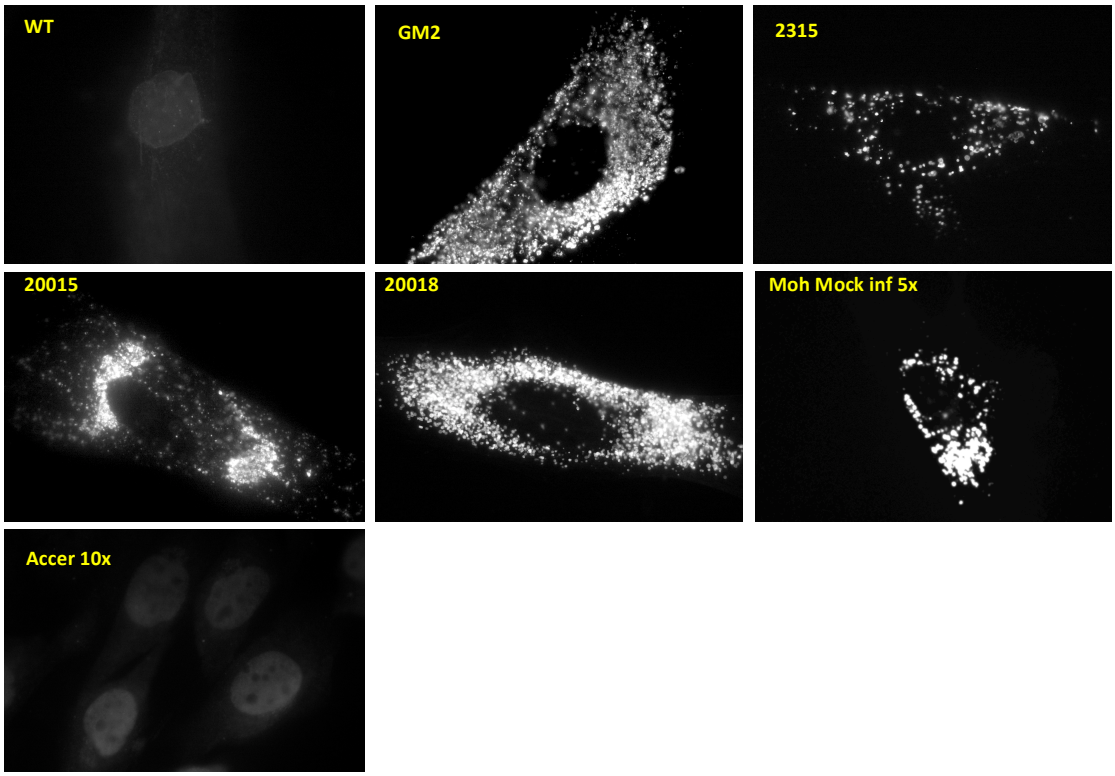

Figure 13 – Evaluation of secondary GSL accumulation in FD cells.

Immunofluorescence analysis of ganglioside GM2 using an antibody anti-GM2 was performed in WT, FD cell lines (2315, 20015, 20018 and Moh Mock inf 5x) and the corrected version Accer 10x. GM2-gangliosidosis B1 variant was used as a positive control. The cells were imaged with a Nikon Eclipse E400 fluorescence microscope (λexc = 494 nm; λem = 518 nm) and charge-coupled device Nikon Coolpix 950 camera. Representative images of n=30 cells are shown.

Comparison between the positive control cells GM2-gangliosidosis and Farber fibroblasts 2315 (Type 3), 20015 (Type 1), 20018 (Type 1) and Moh Mock inf 5x (Type 4), suggest an accumulation of GM2, as shown by the strong labeling with the antibody anti-GM2 (see figure 13). This was not observed in WT and FD corrected cell line Accer 10x, indicating that there is no GM2 accumulation in normal fibroblasts. The punctate labeling pattern observed in FD fibroblasts suggests that the GSL is accumulated in the endosomal/lysosomal system when ACDase is deficient. Interestingly, FD cell line 2315, which corresponds to the less severe mutation, seems to display less GM2 accumulation suggesting a correlation between disease severity and secondary lipid accumulation.

Secondary accumulation of gangliosides might be related to a Chol disturbed homeostasis. This would explain the intralysosomal trapping of GM2 in ACDase-deficient fibroblasts. Therefore it is important to evaluate if Chol homeostasis is also altered in FD fibroblasts.

3.5. Cell Chol imbalance by Filipin staining

An important issue with regard to several lysosomal storage diseases is actually the coordination of Chol metabolism with that of SM and of GSLs. Conversely, lysosomal accumulation of unesterified Chol secondarily affects SM metabolism (Walkley and Vanier, 2009). Not so far, it has further been shown that Chol plays a major part in regulating traffic of SLs along the endocytic pathway (Puri et al., 1999; 2001). Indeed, lysosomal Chol overload correlates with abnormalities observed in NPC cells, but quite unexpectedly, these studies also showed that Chol homeostasis was perturbed in a number of sphingolipidoses, secondary to SL accumulation (Vanier et al., 1991; Walkley and Vanier, 2009).

In several LSD high cellular Chol level was demonstrated to block Golgi targeting of BODIPY-LacCer while Chol depletion of LSD cells restored Golgi targeting of these lipids (Robinson and Karnovsky, 1980; Pagano et al., 2000a). The accumulation of endogenous lipids induces a redistribution of Chol which, in turn, leads to an alteration in the intracellular sorting of GSLs endocytosed via caveolae (for some SL such as SM a clathrin-dependent pathway can also be used to transport the lipid molecule to Golgi, however this pathway is not modulated by cellular Chol) (Puri et al., 2001).

It is known that the incubation of fibroblasts derived from patients with NPC disease with LDL results in excessive intracellular accumulation of unesterified Chol (Kruth et al., 1986; Vanier et al., 1991; Abdul-Hammed et al., 2010).

For the reasons described above, it seems that it is important to study cell Chol imbalance in cells from FD patients.

Filipin is a polyene antibiotic and has been employed as a probe in biophysical studies of membrane structure, binding very specifically to certain sterols (usually Chol) in biological membranes (Robinson and Karnovsky, 1980; Vanier and Millat, 2003). For this reason, filipin complex is commonly used in the diagnosis of NPC patients (De Winter et al., 1992; Cenedella, 2009). To evaluate alterations in Chol traffic, the cells were thus stained with this polyene antibiotic. FD cells cultured in the presence of LDL showed filipin staining in perinuclear granules reflective of lysosomal Chol accumulation (Figure 14), implying that Farber cells are not capable of an efficient metabolism of free cholesterol. The positive control NPC classic cell lines showed a high lysosomal Chol accumulation (Figure 14) even without LDL overloading (not shown). Chol accumulation was not observed in cells overexpressing ACDase (Accer 10x), even when these cells were overloaded with LDL, showing that restoring ACDase activity corrects Chol metabolism. These results are similar to the control WT cells.

These results suggest that a proper function of ACDase is necessary to maintain the Chol homeostasis (normal level of correctly distributed cell Chol) (Figure 14).

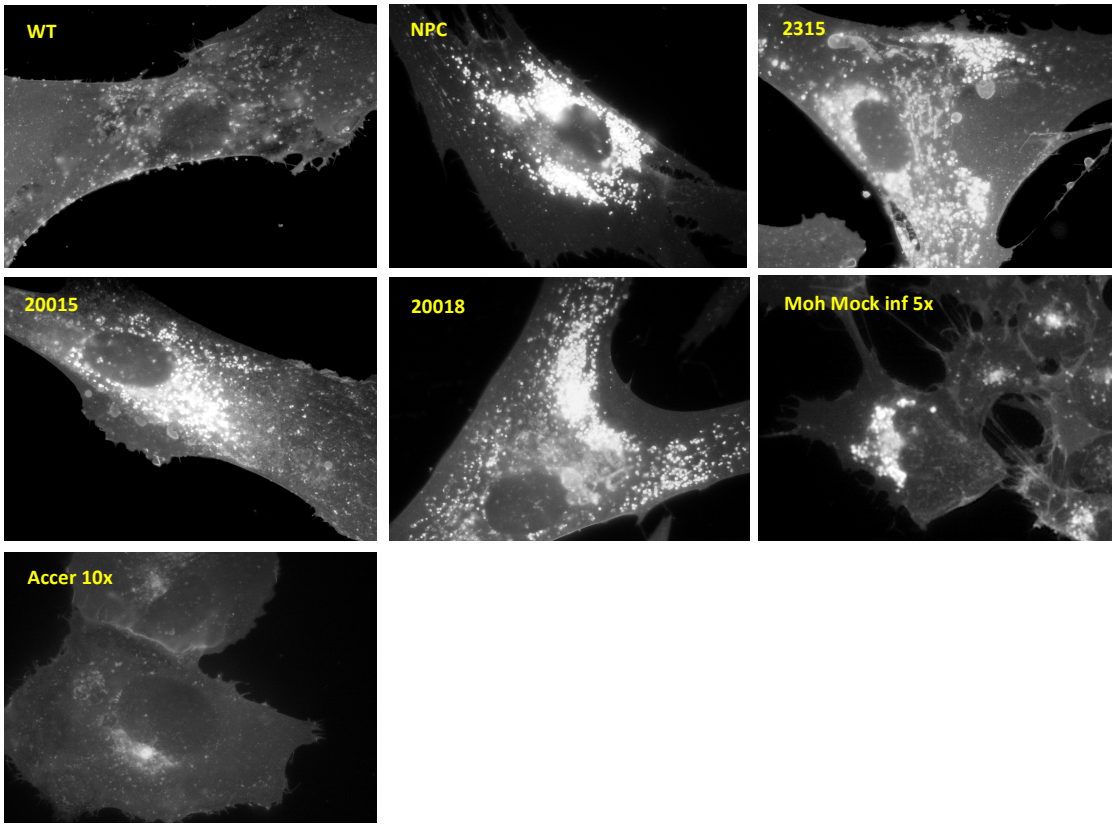

Figure 14 - Cell Chol imbalance by Filipin staining.

The cells were overloaded with LDL (100µg/ml) for 24-h as described in Materials and Methods and visualized with a Nikon Eclipse E400 fluorescence microscope (λexc. = 364 nm; λemi. = 475 nm). WT- normal HSF; NPC- positive control; 2315 (Type 3), 20015 (Type 1), 20018 (Type 1) and Moh Mock inf 5x (Type 4)- ACDase-deficient cells; Accer 10x - cells overexpressing ACDase. T Representative images of n=30 cells are shown.

4. Disruption of Chol and SL metabolism and trafficking in FD

To further confirm the accumulation of Chol as a secondary lipid in FD, another strategy was employed. This strategy is based on literature evidence that shows an accumulation of Chol as a result of decreased activity of ASMase, as observed in NPB (Cenedella, 2009; Abdul-Hammed et al., 2010). As shown in Figure 15, filipin staining in NPB is increased compared to control cells and confirms an accumulation of Chol comparable to NPC cells.

We further investigated the effects of both Chol synthesis/transporter inhibitor and ASMase inhibitor drugs on ACDase and ASMase levels. To induce Chol accumulation U18666A was used, while ASMase activity was blocked by the use of desipramine. Our findings support the hypothesis that perturbation of Chol transport with U18666A influences ASMase activity in Farber, NPC and control cell lines. Our studies have also demonstrated that there may be a relationship between the decrease of ASMase activity and Chol accumulation. This was proven through the filipin staining of NPB cell lines, which carry a primary defect on ASMase, demonstrating a significant accumulation of free Chol (see figure 15).

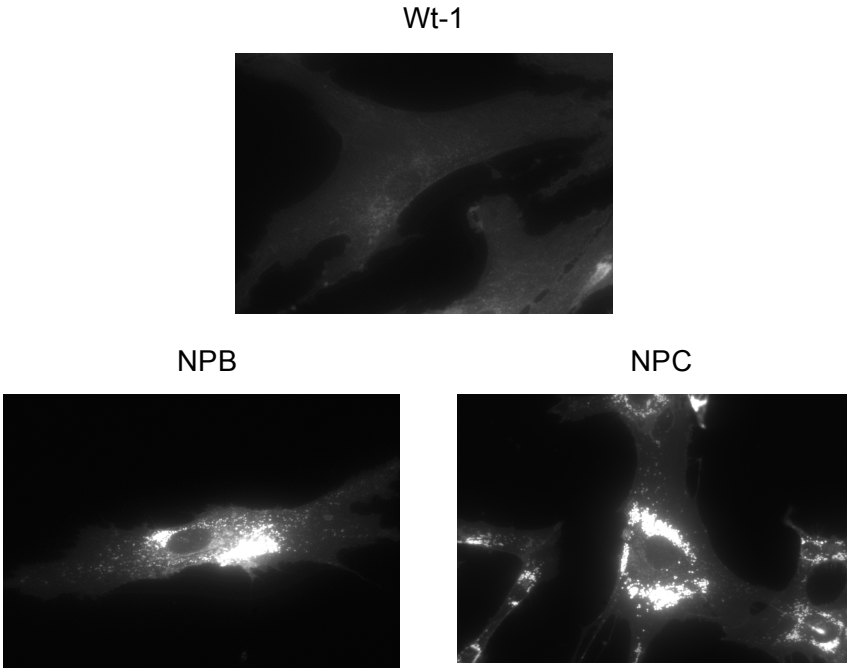

Figure 15 - Free Chol accumulation in NPC and NPB.

The cells were overloaded with LDL (100μg/ml) for 24-h as described in Materials and Methods and visualized with a Nikon Eclipse E400 fluorescence microscope (λ_{exc} = 364 nm; λ_{em} = 475 nm). WT - normal HSF; NPB, NPC - positive control. Representative images of n=30 cells are shown.

Furthermore, when treating normal control cell lines with an ASMase inhibitor (desipramine) and U18666A, we also observed Chol accumulation (see figure 16) together with the expected decrease of ASMase activity (see figure 17).

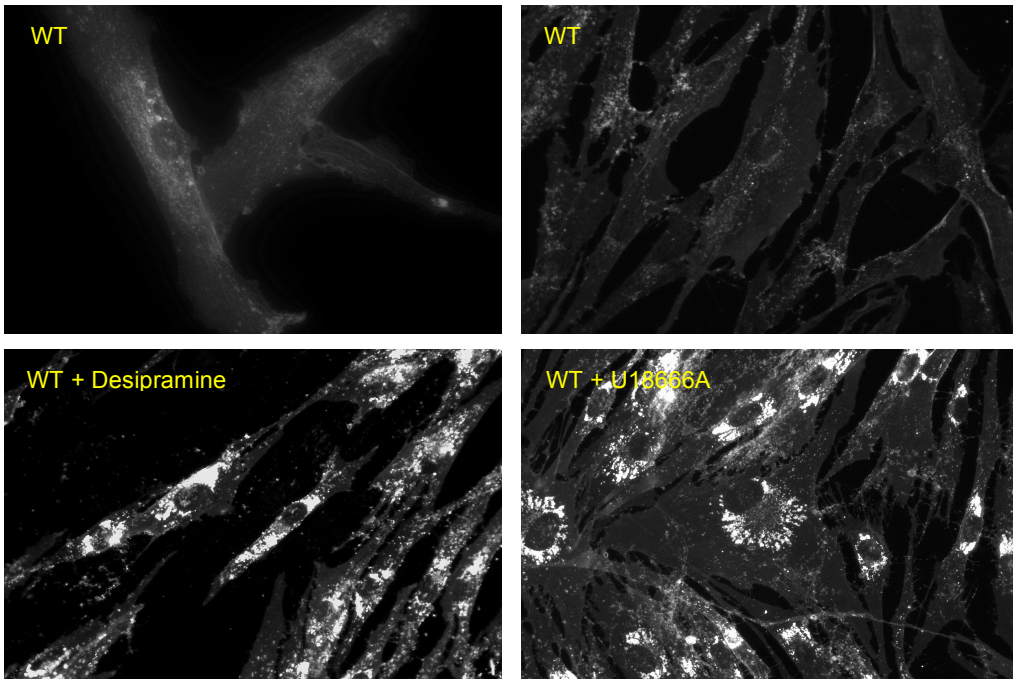

Figure 16 - Study of the effect of desipramine and U18666A in free Chol cell trafficking.

Normal fibroblasts (WT) were grown in medium with or without Desipramine and U18666A for 24 h. Cells were imaged with a Nikon Eclipse E400 fluorescence microscope (λexc. = 494 nm; λemi. = 518 nm) and charge-coupled device Nikon Coolpix 950 camera. Representative images from n=30 cells, are shown.

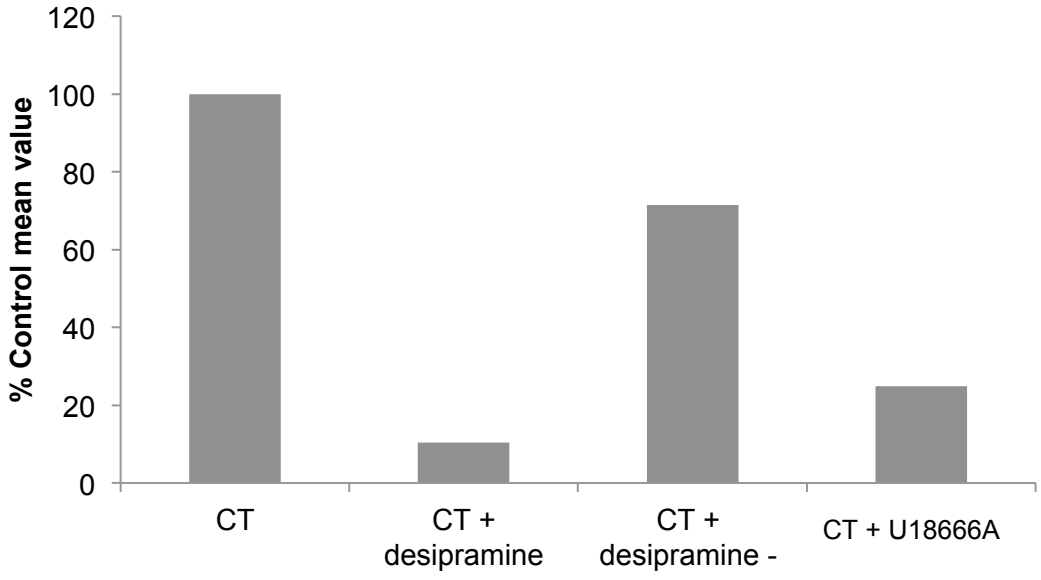

Figure 17 - Effects of desipramine and U18666A in aSMase activity of control cells.

Normal fibroblasts were grown in medium with 4,5µg/ml of U18666A for 24 h, and 3,8µM of desipramine for 24h at 37ºC. CT- Negative control; CT+desipramine – negative control was incubated with desipramine; CT+desipramine -; negative control after incubation with desipramine, and replaced for DMEM during 24h; CT+U18666A- negative control after incubation with U18666A.

Previous studies showed accumulated unesterified Chol in perinuclear cytoplasmic inclusions in fibroblasts from NPC patients (Vanier and Millat, 2003). This pattern can also be observed after LDL overloading causing excessive amounts of predominantly unesterified Chol accumulated (the same observed in Farber cells) (see figure 14).

Initially, normal fibroblasts, Farber, and NPC fibroblasts were grown in medium with or without human LDL and U18666A for 24 h. In control cells unesterified Chol levels are low because the cell has the capacity to esterify of exogenous Chol. Treatment of normal cells and Farber fibroblasts with U18666A promotes an increase in Chol levels due to inhibition of Chol biosynthesis in agreement with previous reports (Cenedella, 2009). Removal of U18666A after 48 hours reverts the NPC phenotype induced by the inhibitor in these control cells, and decreased the accumulation of Chol (Figure 18). In the positive control cells, i.e., NPC cells, filipin staining revealed that Chol levels were already high in untreated cells, as expected due to the intrinsic accumulation of Chol in NPC cells (Figure 18). Treatment of NPC cells with U18666A enhanced the accumulation of Chol, in agreement with previous studies (Cenedella, 2009). Removal of U18666A did not revert the phenotype and accumulation of Chol was still present in these positive control cells (Figure 18). In untreated FD cells, staining with filipin revealed increased levels of Chol compared to negative control cells, suggesting the accumulation of this lipid in FD cells. Treatment with U18666A further increased Chol levels and removal of the inhibitor did not revert the phenotype further supporting the intrinsic accumulation of Chol in FD cells. These results indicate that in addition to Cer accumulation, Chol is also accumulating in FD cells.

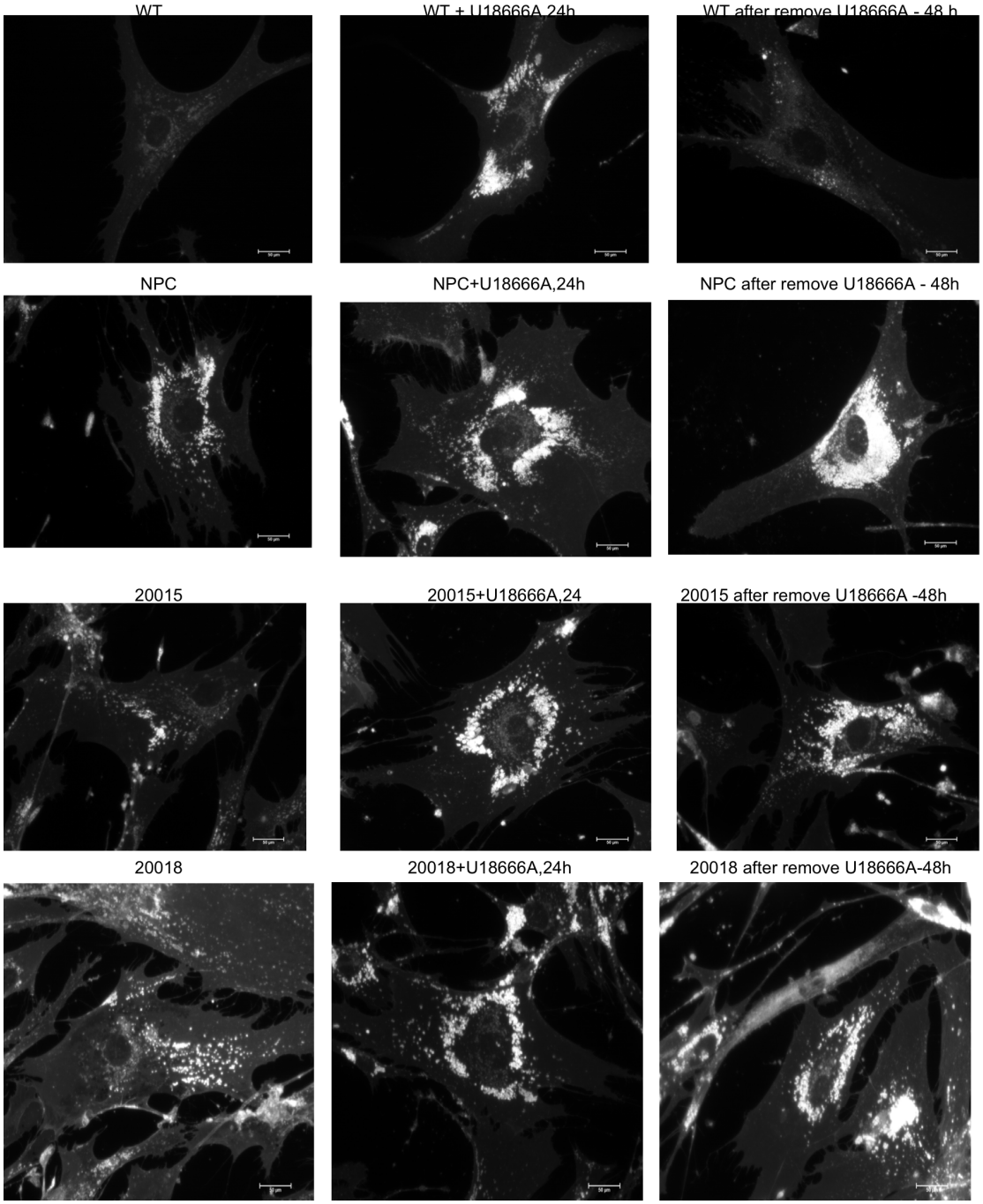

Figure 18 - Study of the effect of U18666A in free Chol cell trafficking.

Normal fibroblasts (WT), Farber (20015 (Type1); 20018 (type 1), and NPC fibroblasts were grown in medium with (first column) or without (second and third columns) human LDL and U18666A for 24 h (second column). The third column represents the replacement for DMEM, and leaved for 48h. Cells were imaged with a Nikon Eclipse E400 fluorescence microscope (λexc. = 494 nm; λemi. = 518 nm) and charge-coupled device Nikon Coolpix 950 camera. Representative images from n=30 cells, are shown.

To induce NPB phenotype in FD and negative control cells, cells were treated with desipramine, which is an inhibitor of ASMase (Elojeimy et al., 2006). Treatment of negative control cells resulted in an accumulation of Chol (figure 19). This was reverted by removal of the inhibitor (figure 19 third column). In positive control cells, i.e., NPC cells, removal of the inhibitor did not revert the accumulation of Chol. Similar results were obtained in FD cells, as above mentioned untreated cells displayed increased filipin staining which was enhaced by the addition of ASMase inhibitor. Removal of the inhibitor did not revert Chol accumulation. These results further support the secondary accumulation of lipids in FD, particularly Chol.

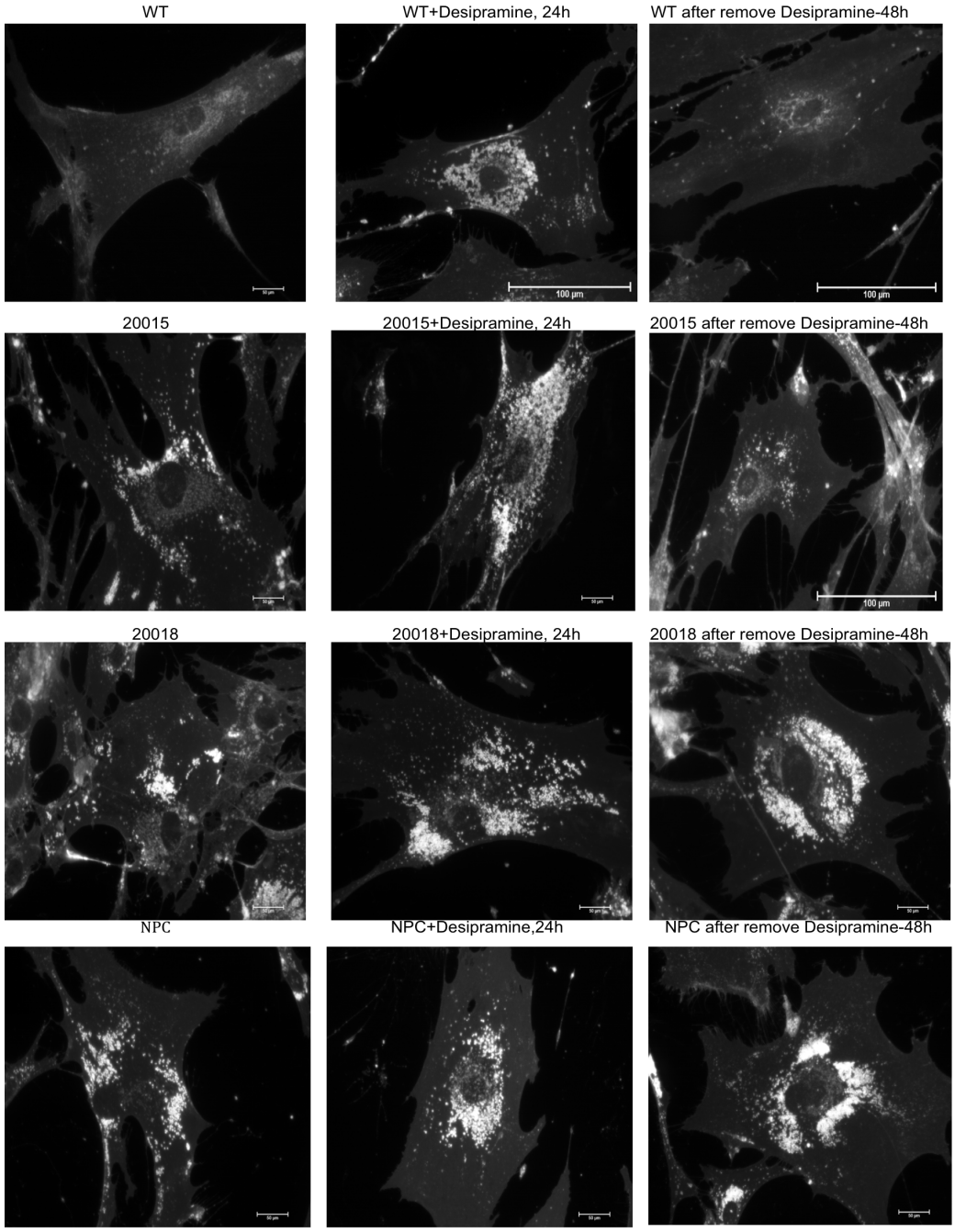

Figure 19 - Study of the effect of desipramine in free Chol cell trafficking.

Normal fibroblasts (WT), Farber (20015 (Type 1); 20018 (Type 1), and NPC fibroblasts were grown in medium with (first column) or without (second and third columns) human LDL and desipramine for 24 h (second column). The third column represents the replacement for DMEM, and leaved for 48h. Cells were imaged with a Nikon Eclipse E400 fluorescence microscope (λexc. = 494 nm; λemi. = 518 nm) and charge-coupled device Nikon Coolpix 950 camera. Representative images from n=30 cells, are shown.

5. Identification of Cer/Chol "lipid domains" in FD fibroblasts

The results presented above show that FD cells display a primary accumulation of Cer and a secondary accumulation of Chol and GM2. It was recently reported that Cer and Chol can coexist in the same domains (Castro et al., 2009) and form structured ordered domains in cell membranes (Goldschmidt-Arzi et al., 2011). This raised the hypothesis that these Cer/chol-enriched domains could exist in FD cells at the place where these lipids accumulate. To test this hypothesis, studies with an antibody able to recognize Cer/Chol domains were performed. This antibody 405F is enabled to recognize Chol/C16-Cer-mixed ordered domains (Scheffer et al., 2006). The Cer–Chol 405F specificity has been extensively studied (Scheffer et al., 2005; 2006; 2007; Addadi et al., 2008; Goldschmidt-Arzi et al., 2011). Previous studies concluded that the binding specificity of this antibody depends on the acyl chain length of the Cer involved in the formation of these structured domains (Scheffer et al., 2005; 2006). It was shown that Cer–Chol 405F binds equally to monolayers composed of 60:40 mol % Chol/C16-Cer and 60:40mol% Chol/C18-Cer, but the binding affinity is decreased if ceramide has a longer acyl chain, e.g., in monolayers composed of 60:40 mol% cholesterol/C20-ceramide (Scheffer et al., 2006; 2007). In the present study it was further used as a control a monoclonal antibody-58B1, which binds to Chol crystal surfaces and to artificially Chol-enriched cell membranes, but not to individual Chol molecules (Scheffer et al., 2006; 2007; Goldschmidt-Arzi et al., 2011).

To evaluate the formation of Cer/Chol structured domains all fibroblasts of WT and different subtypes of FD patients were grown on cover slips, then were fixed and labeled with anti C16 Cer/Chol antibody. The cells were viewed and Image capture by confocal microscopy and their intensity was evaluated by Olympus Fluoview FV1000 software. The intensity was integrated and normalized over cell area. Some labeling could be detected in WT fibroblasts, with a similar distribution to that observed in a number of other cell types (Goldschmidt-Arzi et al. 2011), In contrast, substantially more intense labeling was observed in fibroblasts derived from a type 4 FD patient (Table 5), suggesting that C16-Cer/Chol domains accumulate in FD fibroblasts. Levels of C16-Cer/Chol domains were subsequently quantified in fibroblasts from FD patients of different severity. The most severe patients, namely the three type 4s and one type 7, displayed the highest level of labeling (Table 5), which was ~3-5-fold higher than in control fibroblasts. Fibroblasts derived from patients who displayed a less severe

disease, or a patient who was a FD carrier, did not show elevated levels of C16-Cer/Chol domains (Table 5).

Table 5 - Intensity of Cer/Chol domains in fibroblasts of Farber patients.
Fibroblasts of WT and Farber patients were fixed and labeled with anti C16 Cer/Chol antibody. Acquisition was performed using a confocal FV1000 on Olympus IX81 microscope, using 1.35 NA UPLSAPO 60x oil objective ($\lambda_{exc.}$ = 490 nm; $\lambda_{emi.}$ = 525 nm). The intensity was integrated and normalized over cell area. The Farber fibroblasts are annotated by the age of death and subtype. Data shown are means ± SE (n=50 cells).

NIGMS human genetic cell repository designation	Age at death	Presumed Farber type	Anti-C16-Cer/Chol Fold change (*labeling intensity, arbitrary units*)
WT[1-4]	20-32 years	None	1,00 ± 0,10
20018	22 months	1	0,42 ±0,12
20015	24 months	1	1,19 ± 0,08
2314	6 years	2-3	0,65 ± 0,13
2315	30 years	3	1,22± 0,13
5752	6 months	4	4,75 ± 0,06
18313	11 months	4	5,08 ±0,08
20017	3 years	5	0,35 ± 0,12
20016	29 months	6	0,42 ± 0,12
Sap	4 months	7	2,79 ± 0,12
2316	37 years	None	0,86 ± 0,11
Moh Mock inf 5x	3 days	4	6,94 ± 0,07
Accer 10x	3 days	4	0,95 ± 0,11

We next determined whether levels of C16-Cer/Chol domains could be altered either by modifying Cer or Chol levels.

For the former, we used human simian virus 40 (SV40) large T-transformed skin fibroblasts derived from a patient with type 4 Farber disease (Moh Mock inf 5x), which had been transduced with a recombinant retroviral vector containing the *ASAH1* cDNA (Accer 10x) (Medin et al. 1999). As expected, the untreated cell line (Moh Mock inf 5x)

showed high levels of C16-Cer/Chol domains (Figure 20) whereas levels of the domains were reduced by ~7- fold in the ACDase-ovexpressing cells (Accer 10x) when compared to Moh Mock inf 5x (Table 5).

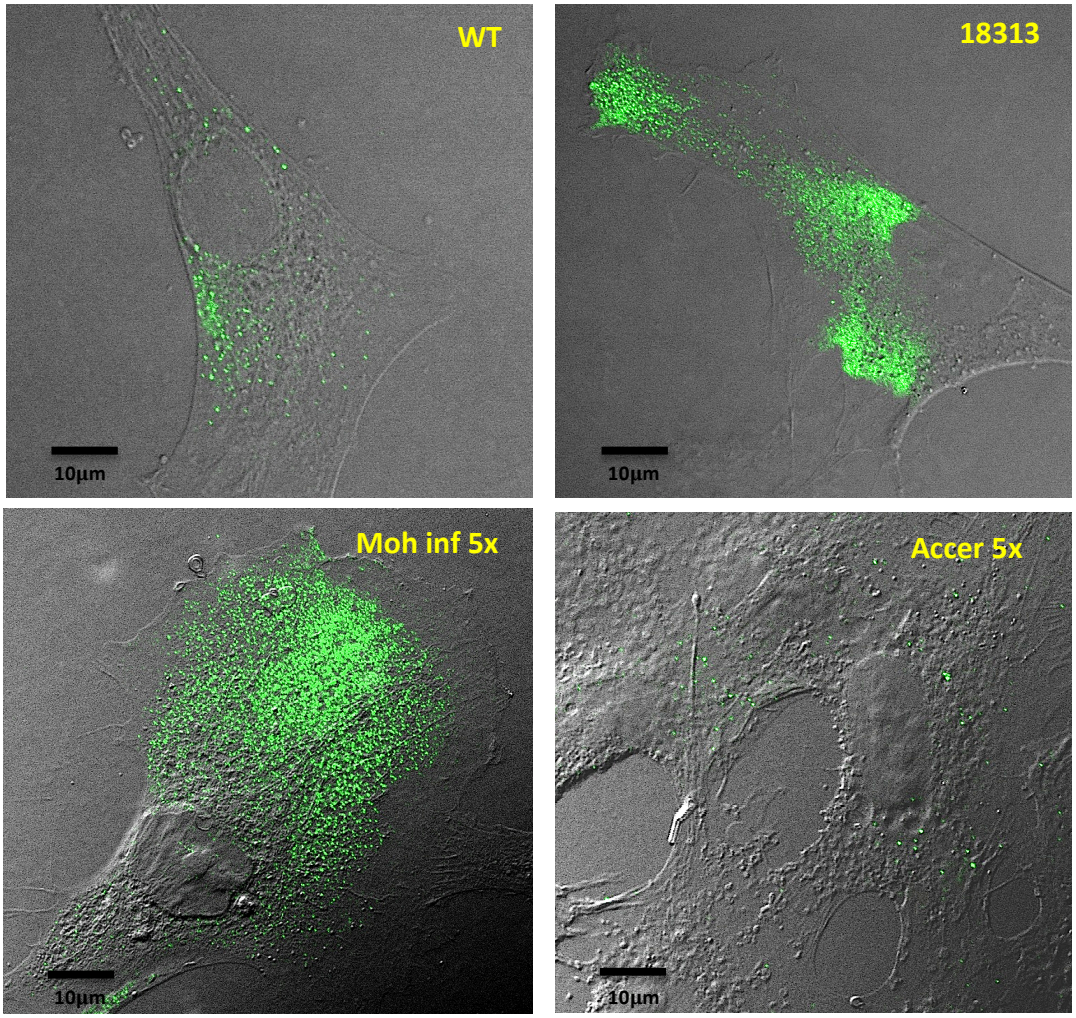

Figure 20 -Confocal immunofluorescence microscopy of HSF stained with anti-C16-Cer/Chol antibody.

Cells were incubated with anti-C16-Cer/Chol antibody as described in Materials and Methods and observation under the confocal fluorescence microscope FV1000 on Olympus IX81 microscope, using 1.35 NA UPLSAPO 60x oil objective (λexc. = 490 nm; λemi. = 525 nm). Images of fibroblasts labeled with C16 Cer/Chol antibody, negative control (WT), Farber type 4 (18313), Farber type 4 (Moh Mock inf 5x) and Retroviral corrected cells (Accer 5x). Cer/Chol domains decrease after ACDase retroviral correction. Magnification=×40.

To further evaluate the correlation between lipid accumulation in FD and the amount of Cer/Chol domains, 5752 (Type 4) Farber fibroblasts were treated with CD in order to decrease the levels of Chol, as previously reported (Kilsdonk et al., 1995). This macromolecule is able to sequester Chol from the membranes (McCauliff et al., 2011). A significant decrease, ~6 fold, in the intensity of the C16-Cer/Chol domains was

observed n FD fibroblasts, with no effect on levels of the domains in WT fibroblasts (Figure 21).

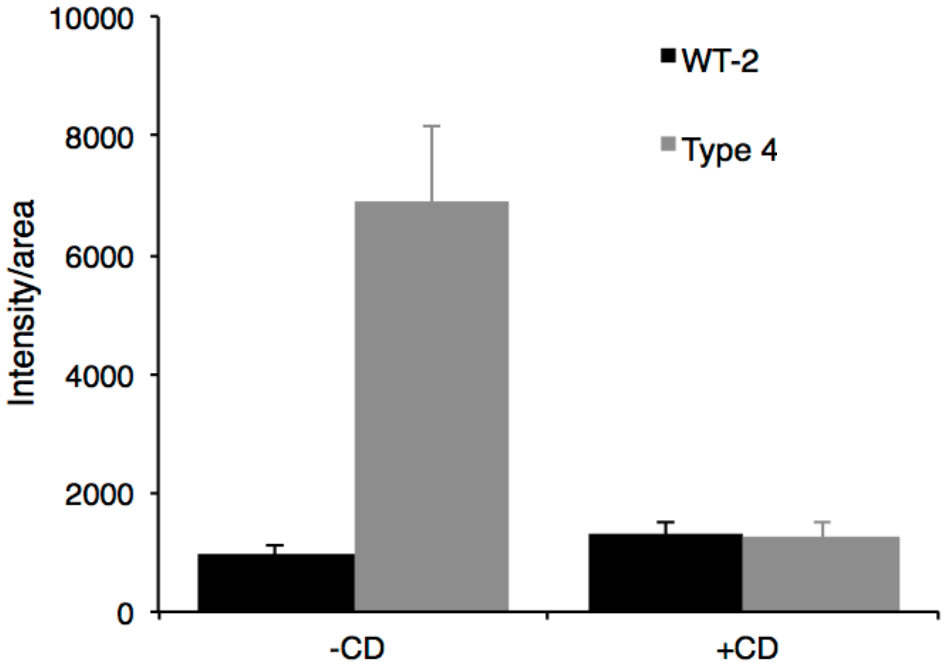

Figure 21 - Effect of CD treatment on anti-C16-Cer/Chol labeling.

Domain intensity was measured in WT-2 and 5752 (Type 4) untreated fibroblasts and after treatment with 100μM CD for 48h. Values are means ± s.e.m., n = 2.

6. Cellular localization of Cer/Chol cell domains in FD

The intracellular location of the C16-Cer/Chol domains was further examined by co-localization with a number of organelle markers. As observed in earlier studies (Goldschmidt-Arzi et al. 2011), significant co-localization was observed in membranes of the endocytic pathway, including early and late endosomes and lysosomes but, unexpectedly, a significant increase in domain labeling was also observed in both the mitochondria and in the PM of type 4 Farber disease fibroblasts (Figure 22).

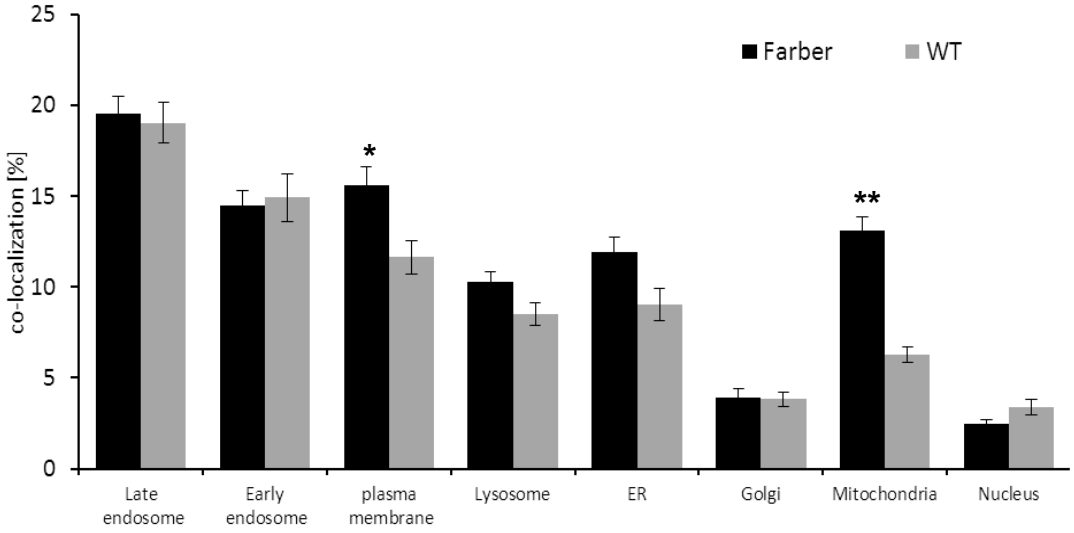

Figure 22 -Cellular localization of C16-Cer/Chol domains.

The extent of co-localization between the anti-C16 Cer/Chol antibody and each of the markers was determined in WT-2 and Type 4 fibroblasts. Values represent the extent of co-localization within one pair. Data are means ± s.e.m., n = 2. (*p < 0. 01, ** p < 0.0001).

This localization was confirmed by immunogold labeling and TEM, in which significant labeling was detected in the mitochondria and in the PM (Figure 23). As for immunofluorescence microscopy, cells were fixed but not permeabilized, and labeled with the anti-C16-Cer/Chol antibody, followed by a secondary antibody conjugated to 1.2 nm gold and by gold enhancement (Figure 23). Upon analyses in TEM the Cer/Chol domain labeling in the Farber type 4 samples in the mitochondria (Figure 23-D) and PM 23-B) were viewed at higher quantity while in the WT fibroblasts sporadic gold particles were seen in both PM (Figure 23- A) and in the mitochondria 23-C).

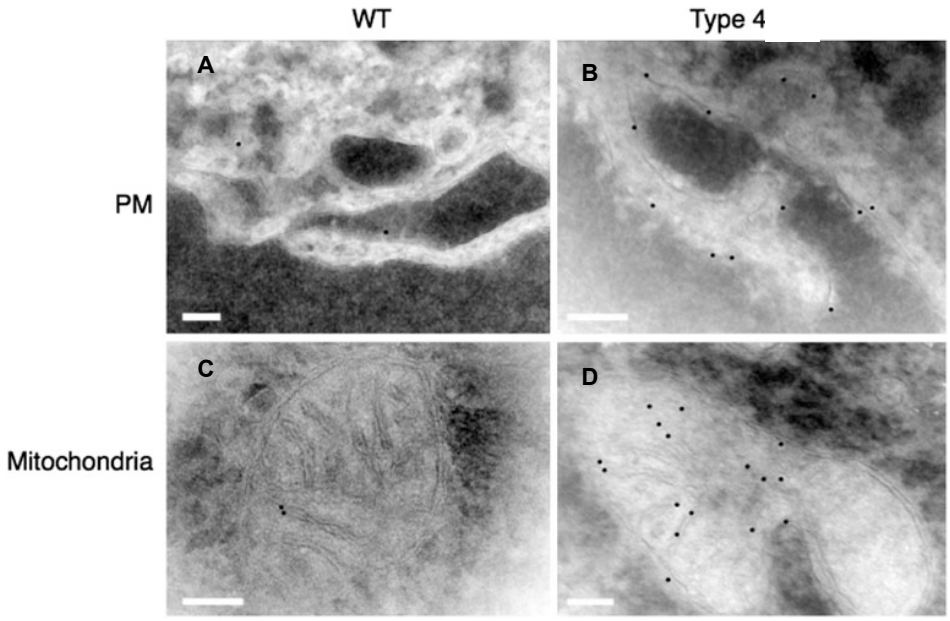

Figure 23 - Visualization of Cer/Chol domains in the Plasma membrane (PM) and mitochondria by electron microscopy.

Farber fibroblasts type 4 (5752-6m) and WT were labeled using the anti-C16 Cer/Chol antibody and a gold-conjugated secondary antibody. In the TEM images of WT/ Farber fibroblasts gold particles are seen as small black dots. WT PM (A) Farber PM (B) WT mitochondria (C) Farber mitochondria (D); (gold particles are indicated as magnified black dots); Scale 100 nm.

7. Cer/Chol domains are absent in other LSDs

To understand if the increase in the occurrence of Cer/Chol domains is a phenomenon common among LSD's we grew fibroblasts of WT and different LSD patients with representative Farber fibroblasts on cover slips. The cover slips were fixed and labeled with anti C16 Cer/Chol antibody. The cells were imaged by confocal microscopy and their intensity was evaluated by Olympus Fluoview FV1000 software. The intensity was integrated and normalized over cell area (Figure 24).

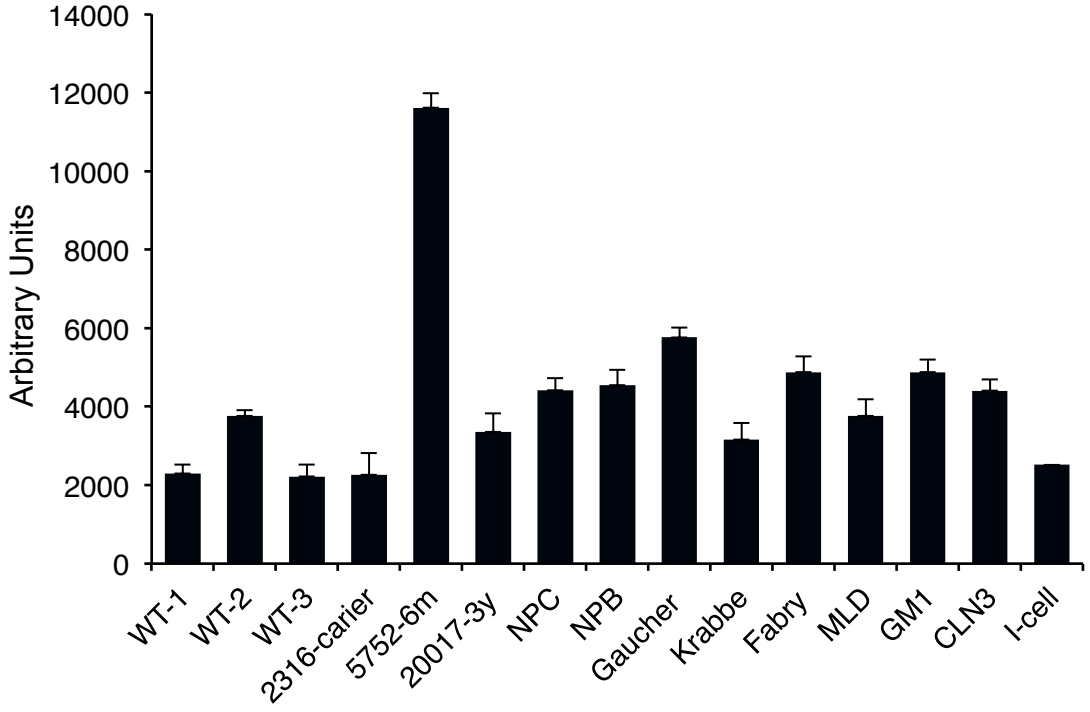

Figure 24 - Intensity of C16 Cer/Chol domains in fibroblasts from patients with different LSDs.

Fibroblasts of WT and LSD patients were fixed and labeled with anti C16 Cer/Chol. The cells were visualized under the confocal fluorescence microscope FV1000 on Olympus IX81 microscope, using 1.35 NA UPLSAPO 60x oil objective ($\lambda_{exc.}$ = 490 nm; $\lambda_{emi.}$ = 525 nm). The intensity was integrated and normalized over cell area. Data shown are means ± SE (n=50).

In all LSD fibroblasts used for this assay only one patient sample were used for each disease, therefore conclusions should be drawn carefully and relate to the specific subtypes of the disease presented only. The Cer/Chol domain elevation was not evident in any of the other LSD's examined here, when compared to Farber cell line from subtype 4 (5752) (Figure 24). When evaluating the Cer/Chol domains labeling in the LSD fibroblasts we saw that only in Gaucher the elevation is slightly higher comparing to the WT and others LSDs. In Gaucher type 2 fibroblasts the cells showed a ~2 fold increase in Cer/chol domains intensity. However this result needs statistical confirmation, once in this study, it has been used only one Gaucher cell line.

Discussion

Discussion

Farber disease is an autosomal recessive storage disease caused by different mutations reducing or eliminating ACDase activity and leading to the accumulation of Cer in lysosome. The disease appears in heterogeneous population with no specific ethnicity or gender. As a result Cer accumulates in many different tissues presenting several different storage bodies some common to other LSD's and some unique Farber bodies (Schmoeckel and Hohlfed, 1979). The characterization of which lengths of Cer are accumulated among patients was never performed. The substrate specificity of the ACDase was studied, greater activity of ACDase in pH 4.5 was found when the Cer substrates containing shorter-chain fatty acids (C12-C18) or higher content of double bonds was seen (Momoi et al., 1982). Since these experiments were performed in vitro and involved the use of detergents a direct deduction to the biological system cannot be done. The main group of ceramides distributed in skin fibroblasts was identified as C22-C24 being the major species (Goto-Inoue et al., 2012). Usually the diagnosis is made by measure the ACDase activity in skin fibroblasts. However the procedure is not so simple, once to obtain the protein amount take some time, and the diagnosis cannot be done so fast. For this reason becomes interesting and useful to do other studies, including secondary lipid accumulations, that help us to understand the mechanism of disease.

1. Secondary lipid accumulation in Farber Disease

A relatively large subgroup of lysosomal storage diseases are caused by defects in enzymes and proteins required for lysosomal catabolism leading to accumulation of lipids in late endosomes or lysosomes (Simons and Gruenberg, 2000). An example already well studied, is NPD, which has more than one cause; NPA and NPB are characterized by SM accumulation, whereas Chol typically accumulates in the third form, NPC. The last one, that has accumulation of Chol in degradative compartments of the endocytic pathway is apparently due to a failure in the mechanism responsible for redistribution of Chol taken up by endocytosis of LDL, in contrast to other storage diseases caused by defective metabolic enzymes (Simons and Gruenberg, 2000). However previous studies have shown that, in addition to the major defect in Chol transport in NPC, SLs also accumulate in the multivesicular compartments of the endocytic pathway (Simons and Gruenberg, 2000).This is true not only for the NP diseases, since other SL storage diseases accumulate different SL classes in late endosomes/lysosomes. It turns out that cells from patients with sphingolipidoses also exhibit increased Chol levels in endocytic organelles (Simons and

Gruenberg, 2000). Indeed, it has been shown that endogenous lipids accumulate in the different LSD fibroblasts as a result of the primary defect in lipid catabolism, which then affect the intracellular distribution of Chol. This, in turn, leads to alterations in membrane composition that results in defective sorting and transport, which is typically monitored by following the trafficking of fluorescently-labeled SL analogs. Alterations in membrane trafficking and secondary lipid accumulation seem therefore widely common phenomena in many LSDs. However, this issue has so far not been addressed in FD, which prompted the investigation of putative secondary lipid accumulation subsequent of intralysosomal accumulation of Cer in fibroblasts from FD patients carrying different mutations. In addition, the studies performed in the framework of this thesis aimed to investigate if alterations in lipid trafficking and secondary lipid accumulation could be correlated with the severity of the disease. It has been described that secondary lipid accumulations in LDSs can belong to any of the major classes, Chol, phospholipids and GSLs, with different patterns in the different tissues often occurring in the same disease (Walkley and Vanier, 2009). Accordingly, studies with fluorescent (BODIPY) SL analogues were performed in ACDase-deficient fibroblasts, to evaluate the biological impact of the intralysosomal accumulation of Cer on other cellular compartments and on secondary lipid accumulations, such as Chol and ganglioside GM2.

Pagano and collaborators contributed greatly in this type of studies when used fluorescent derivative of lactosylceramide, a glycosphingolipid carrying the fluorescent BODIPY moiety in the fatty acid amide bonded to sphingosine. The authors have shown that the trafficking of this analog is disturbed in both Chol and SL storage disorders (Chen et al., 1999; Pagano et al., 2000). It is known that, in normal fibroblasts, after endocytosis at the PM, both SLs and Chol predominantly recycle to the PM (either directly or via the Golgi apparatus), possibly within SL/Chol enriched microdomains (Pagano et al., 2000). BODIPY-LacCer would partition into SL/Chol enriched microdomains at the PM or in endosomes, and the presence of high levels of SLs and Chol in LDSs would cause an increased fraction of SL/Chol enriched microdomain units to be shunted to the degradative pathway, possibly as a mechanism to regulate PM composition (Pagano et al., 2000). These phenomena could occur by exceeding the capacity of the recycling machinery by lipid environmental effects on proteins involved in sorting or by changes in ‘membrane architecture’. For instance, altered lipid composition could affect the curvature of nascent vesicles or tubules causing them to be recognized by different sorting proteins. Morphologic observation of lateral segregation of endosomal membranes into domains with higher and lower concentrations of BODIPY- LacCer would be consistent with this model (Pagano et al., 1991). The concentration-dependent spectral properties of the fluorophore BODIPY allow the identification of regions poor or enriched in the lipid analog,

as described in the results section, and thus, the identification of alterations in lipid trafficking. The model proposed by Pagano and collaborators supports the qualitative observations shown in figure 11, which demonstrate that BODIPY-LacCer accumulates in the lysosomes of the NPC, 20015, 20018 and Moh Mock inf 5x fibroblasts. These results are therefore suggestive of alterations in trafficking of SL and/or Chol, which might thus be associated to alterations in the composition of membrane microdomains and/or membrane properties. Such alterations were not observed in normal and 2315 fibroblasts. In these cells, BODIPY-LacCer labeling was mainly observed in the reticular and perinuclear region corresponding to the Golgi complex, in contrast to the punctate distribution observed in NPC, 20015, 20018 and Moh Mock inf 5x fibroblasts. 2315 corresponds to a mild form of FD, characterized by mental retardation and muscle weakness, the age at death was 30 years old. At the molecular level, these cells have a significantly higher ACDase activity, when compared to the severe and very severe forms of FD patients. This certainly has a lower impact on the trafficking and accumulation of other lipid species compared to fibroblasts where the activity of ACDase is residual and Cer accumulation is elevated, such as observed in the classical (20018, 20015) and, neonatal (Moh Mock inf 5x) forms of the disease. Further correlation between alterations in BODIPY-LacCer trafficking and the activity of ACDase could be established by studies in FD corrected cells, where the reestablishment of the activity of the enzyme corrected for the impaired LacCer trafficking. It can thus be concluded that deficiency in ACDase activity, as observed in FD, has implications not only at the level of intralysosomal Cer accumulation, but also on the trafficking and sorting of GSL, which might be derived from alterations in the composition of SL/chol microdomains, as previously suggested by Pagano and collaborators (Pagano et al., 2000; Pagano, 2003). Moreover, these alterations are dependent on the severity of the disease, being augmented in the more severe cases.

Fibroblasts from NPC patients exhibit metabolic abnormalities in the cellular processing of LDL. All homeostatic responses associated with the cellular regulation of unesterified Chol levels were shown to be delayed in NPC fibroblasts. For this reason LDL-Chol was abnormally sequestered in lysosomes of NPC fibroblasts, which resulted in impaired translocation of Chol to the PM (Distl et al., 2003).

In this study were used two different drugs, U18666A and desipramine. The first one is responsible to cause cellular phenotype similar to NPC cells by oxysterol binding protein (OSBP) dephosphorylation in the Golgi apparatus (Mohammadi et al., 2001). OSBP is a potential Chol regulator or sensor in the Golgi apparatus (Mohammadi et al., 2001).

The second, demonstrated in previous studies to be a specific ASMase inhibitor by induction of intracellular proteolytic degradation of mature ASMase but not other lysosomal enzymes (Elojeimy et al., 2006).

ASMase degrades SM to Cer at the surface on the intraendosomal vesicles. Decrease of SM and increase of Cer enhances the availability of Chol on inner membranes for NPC2 which removes Chol from the inner endosomal vesicles and transfers it to the N-terminal domain of NPC1 in the limiting membrane of late endosomes (Abdul-Hammed et al., 2010). NPC1 mediates Chol exit through the glycocalix. Thus, SM accumulation due to lysosomal ASMase deficiency leads to defective Chol trafficking and efflux, which is believed, is due to sequestration of Chol by SM and possibly other mechanisms (Leventhal et al., 2001).

The altered trafficking of fluorescent SL analogs in LSD fibroblasts is a multistep process and it has been further shown that Chol plays a major part in regulating traffic of SLs along the endocytic pathway (Pagano, 2003). Different studies, (Maxfield and Tabas, 2005) suggested that high concentrations of Chol and SLs that accumulate in the endosomes of NPC cells could perturb endosomal sorting and reduce the amount of SL that can be transported to the Golgi. In this study we examined a fluorescent Cer analogue (BODIPY-C5-Cer) for studying lipid traffic at the Golgi apparatus in FD fibroblasts. Due to the concentration-dependent spectral properties of BODIPY-C5-Cer it was possible to visualize fine tubulovesicular processes extending from the Golgi apparatus (Pagano et al., 1991). The spectral red-shift in the fluorescence emission of the BODIPY-lipid at the Golgi apparatus suggests that BODIPY-C5-Cer and its metabolites were present at this organelle in higher concentrations than in other regions of the cell (Pagano et al., 1991). It is possible that Cer turnover and/or its metabolites, SM and glucosylceramide, is delayed at the Golgi apparatus, further implicating alterations in the membrane composition and function including the distribution of GSLs at the trans-Golgi. Interestingly, it has been reported that SM levels are increased in FD fibroblasts, most likely due to delayed degradation (van Echten-Deckert et al., 1997). This might be a consequence of the sequestration of Cer and its metabolites at the Golgi apparatus, as observed in the present study. Accumulation of Cer in cellular membranes is expected to promote massive changes on membrane biophysical properties, including strong morphological alterations, as tubules, and formation of highly-ordered gel domains (Silva et al., 2007; 2009). These tubules together with the distinctive and highly ordered nature of Cer domains will certainly affect lipid and protein sorting and recycling, but might also modulate the activity of both integral and peripheral membrane proteins, as shown for instance, for SMase and PP2A. Alterations in membrane properties subsequent of Cer accumulation would therefore

globally contribute to the mistrafficking observed in FD fibroblasts and might even constitute one of mechanisms underlying the early stages of the disease.

It has been shown that a secondary accumulation of GM2 ganglioside is a common feature associated with neuropathology in a number of LSDs, such as NPD and mucopolysaccharidoses, but also prosaposin deficiency, as well as some glycoproteinoses and ceroid lipofuscinoses (Walkley, 2004). Immunostaining studies applied to lysosomal diseases undergoing secondary lysosomal accumulation of GM2 have consistently shown that these gangliosides are sequestered in vesicles, appearing as punctate, granular structures within the cytoplasm of cells (Walkley and Vanier, 2009). This labeling has been documented in a wide variety of lysosomal diseases, such as NPA and NPC (both NPC1 and NPC2 deficiencies), MPS diseases including type I, II, IIIA, VI, and VII, mucolipidosis type IV, several of the Batten disorders, including CLN2, CLN6 and CLN10 diseases, and α-mannosidosis (Walkley and Vanier, 2009). Lysosomal Chol overload correlates with abnormalities observed in NPC cells, but, some studies also showed that Chol homeostasis was perturbed in a number of sphingolipidoses, secondary to SL accumulation (Walkley and Vanier, 2009).

We have shown that in fibroblasts from FD patients there is accumulation of both Chol and G_{M2} ganglioside. It can be suggested that, after the primary deficiency in the activity of lysosomal ACDase and accumulation of Cer in cells and tissue, the amount/composition of lipid rafts might be altered in the late endosomes. Previous studies showed that the degradation of SLs is normal when Chol is exiting normally from late endosomes (Walkley, 2004). In the same way, Chol removal from late endosomes would operate normally only when the SL content in the late endosomes is low (Walkley and Vanier, 2009). This phenomenon common to many LSDs, may be occurring in FD patients, as a consequence of the primary Cer accumulation and secondary lipid accumulations observed. Previous observations indicate that in LSDs involving lipids raft, the accumulation of one of the lipid raft components, such as Chol, SM or glucosylceramide, would slowly lead to trapping of other lipids raft components in late endosomes. The abnormal accumulation of any of these lipids would then jam both SL degradation and Chol trafficking (Simons and Gruenberg, 2000). Once Cer has been shown to interplay both with Chol or SM – key components of lipid rafts – and consequently changes the biophysical properties of raft domains (Silva et al., 2007; Castro et al., 2009; Silva et al., 2009) it can be hypothesized that, the large amounts of Cer found in FD patients, might disturb SL/Chol metabolism and trafficking, resulting in their accumulation. The system late endosomes/lysosomes constitute the degradative compartments for SLs. The Cer effects on membrane properties are extremely dependent on membrane lipid composition, especially on Chol amounts. The proper function of this system depends on many factors,

such as the overall load of lipid rafts being endocytosed and recycled, and the balance between Chol and SLs (Castro et al., 2009).

It should be noted that mistrafficking of GM2 as observed in FD fibroblasts, is certainly a consequence of the deficiency in ACDase activity and accumulation of Cer, since reestablishment of Cer levels in corrected FD cells (Accer 10x), and thus with normal ACDase activity, restores the normal distribution of GM2 within the cells.

2. Formation of Cer/chol domains

The biological membranes are constituted by Chol-SLs interactions and they are important for the membrane organization, function, and structure developed (García-Sáez and Schwille, 2010). Since the publication by Singer and Nicolson in 1972 about fluid mosaic model many significant advance in the understanding of membrane organization are made. Many groups suggested introduction a new concept for PM model, once the PM does not exist only as a fluid, (liquid-crystalline phase), but in the PM and some organelles of the secretory and endocytic pathways in eukaryotic cells are rich in SLs, and as a consequence the lipid melting temperature increases, which can change the membrane properties and phase (Holthuis et al., 2001). SM is the most abundant SL present in lipid rafts (Xu et al., 2001), and together with Chol promote the membrane organization. The lipid raft model considers the lipids to be packed in dynamic liquid-ordered domains that are stabilized by specific proteins (Xu et al., 2001). These lipids rafts often referred to as lipid microdomains and result from the dynamic clustering between Chol and SLs (Simons and Ikonen, 1997) playing an important role in membrane trafficking and in signaling as well as pathological processes. Cer can be generated in stress conditions either from SM in rafts or from serine palmitoy-CoA by de *novo* pathway (catalysed by ceramide synthase) (Castro et al., 2009). Previous studies showed that Cer-induced alterations on membrane biophysical properties are dependent on the initial lipid composition, specifically from the Chol content. It is known that increasing Cer concentration leads not only to the formation of large and highly ordered Cer platforms, but also to morphological alterations in the vesicles, including aggregation, vesiculation, and tubular structure formation. Previous studies have shown that Cer-rich gel domains are formed at low but not at high Chol content (Castro et al., 2009). In fact in the presence of Chol, Cer would selectively displace Chol from rafts, competing for association with the other lipids, both in model and in cell membranes (Silva et al., 2007; Castro et al., 2009). In this study was used an antibody, anti-C16-Cer/Chol that was specifically selected to recognize organized arrays of cholesterol and C16-ceramide from a mixed phase with a well-defined structural

organization and does not recognize or bind to arrays composed of Cer or Chol by them selves (Scheffer et al., 2005). Previous studies have shown these domains were located in late endosomes (LEs) and in the trans-Golgi network. The ability of the anti-C16-Cer/Chol antibody to label LEs indicates that free Cer is present in LEs, and that at least some of this Cer is tightly associated with Chol (Goldschmidt-Arzi et al., 2011). This antibody is sensitive to the levels of Chol and Cer, as well as to the acyl chain length of the Cer forming these structured domains (Scheffer et al., 2006). Indeed, it has been shown that when cells were incubated with fumonisin B1, a ceramide synthase inhibitor that promotes a cellular reduction of ceramide, and labeled with antibody Cer–Chol 405F, the fluorescence signal was significantly reduced when compared to untreated cells (Goldschmidt-Arzi et al., 2011). In the contrary, cells overexpressing CerS5, CerS1 or CerS4, which lead to an increase in C16-, C18- and C20-ceramide, respectively, different levels of fluorescence were observed when the cells were labeled with the Cer–Chol 405F. The authors showed that CerS1 and CerS5 transfected cells had much higher levels of fluorescence than control cells, whereas the labeling in CerS4-transfected cells was similar to that of control cells (Goldschmidt-Arzi et al., 2011). This is due to the specificity of the antibody in recognizing structured domains of cholesterol with C16-ceramide/C18-ceramide species (Goldschmidt-Arzi et al., 2011). Due to its characteristics, this antibody was employed as a tool to investigate if the primary accumulation of Cer and secondary accumulation of Chol altered the distribution pattern of these domains in FD cells and if these lipids accumulated together. In addition, the specificity of the antibody towards specific ceramides provides additional insights into the Cer species that accumulate in FD, an issue that has so far been overlooked.

In the Farber fibroblasts we have documented the existence of the cer/chol domains in the two new cell locations, the PM and mitochondria; this finding directly indicates the presence of ceramides C16 and C18 with Chol in these locations. Since the antibody specifically recognizes structures composed of 40/60 mol% Cer/Chol, this is also an indication that the Chol concentration is higher than the Cer at least locally in the specific membrane and might hint that Chol accumulation plays a role in the patient's symptoms. Moreover if this is the case and chol is "trapped" in the cer/chol domains, a reduction of Chol will eliminate Cer/Chol domains increase, and might even improve the cell conditions (Ferreira et al., 2013). Indeed, treatment of the cells with CD, which sequesters Chol from the membrane, resulted in a decrease in the antibody-associated labeling of the cells, to levels comparable to normal cells.

The Cer/Chol domain elevation was not evident in any of the other LSD's examined here, except in the case of Gaucher disease, there seems to be an increase, however these results need to be confirmed.

The existence of C16-C18 ceramides in skin fibroblasts is certain although not as high as longer ceramides, but these species certainly exists and since their metabolism is impaired the accumulated amount is significant for the Cer/Chol domain formation. In light of the antibodies binding site, which is around 20-25 molecules, many molecular recognitions events will happen upon Cer elevation. The domain formation could be a result of different reasons: the intuitive explanation is that domains of Cer and Chol are thermodynamic stable and therefore will form as a result of proximity in a membrane. Since Cer was not so abundant in these membranes in the WT cells the domains did not exist.

This hypothesis, however, does not explain how the Cer arrived to these locations. Another observation that cannot be understood by the abundances of Cer is the lack of Cer/Chol domains increase in other Farber fibroblasts of different subtypes. We would expect to see gradual elevation depending on the severity in other Farber fibroblasts subtypes since Cer is clearly accumulating in them as well. Since this is not the case we propose another explanation with a biological nature. We suggest that the role Cer/Chol domains play in the signaling pathway upon stress is the main reason for the appearance of Cer/Chol domains in the mitochondria and the PM, a possibility worth further consideration in this context. The mitochondrial Cer pool was the focus of various works, which claimed that a Cer pool becomes apparent only when the mitochondria are activated and a stress pathway is initiated (Siskind et al., 2010).

One paper shows Cer generation is independent of the known downstream actions of BAK (BcL2 homologous antagonist/killer) the protein identified as an initiator of apoptosis (via mitochondrial outer membrane permeabilization or caspase protein apoptosis marker activation), suggesting a role for BAK in apoptosis related to Cer with C16 or C18 acyl chains (Siskind and Colombini, 2000; Siskind et al., 2008; 2010).

Another group of papers introduces the idea that C16-Cer, but not dihydroceramide, forms large channels in PM; this action did not trigger cytochrome *C* secretion or release, but simply raised the permeability of the mitochondrial outer membrane, via the Cer channel formation (Siskind and Colombini, 2000).

Several inducers of cellular stress leading to apoptosis have been shown to cause a net increase in cellular Cer levels. This list of inducers causing Cer accumulation suggests that Cer is a crucial component of intracellular stress response pathways (Hannun, 1996). All these accumulating data, converge to the conclusion that Cer is involved in promoting cell stress. This reinforces our belief in the involvement of Cer/Chol domains as an optional biophysical mechanism to change membrane properties/ rigidity in stress cell conditions. Although no definite proof of the direct involvement of Cer/Chol domains in cell stress was

shown, we believe that the existence of the domains in mitochondrial membrane is by itself an important finding.

We also found these new Cer/Chol domains in the PM (Ferreira et al., 2013). The domains in the PM were mostly seen in a proximity to a fusing vesicle and can be thus explained as an exocytosis mechanism in which some surplus lipids are taken out of the cell. This idea is reinforced by the connection found between Cer and exosomes. It was found that cargo taken into endocytotic organelles is segregated into distinct subdomains and the transfer of exosome-associated domains into the lumen of the endosome does not depend on the function of the ESCRT (endosomal sorting complex required for transport) machinery, but requires Cer (Trajkovic et al., 2008). In addition budding of blebs and shedding of vesicles is directly related to the formation of apoptotic bodies, the involvement of Cer as an inducer of this apoptotic process was established in the sphingomyelinase apoptotic pathway (Tepper et al., 2000).

These concepts involving both mitochondria and PM with connection to Cer, clarify that Cer has a big role as structural, biophysical modifier of membrane processes and that the Cer/Chol domains might be an interesting way to control its involvement in Farber pathology.

The mechanism of the Farber pathology and many other LSD's is not clear yet, and the involvement of a genetic background complicates the disease classification. The classification into different subtype emphasizes the different manifestations of the disease symptoms and probably the severance of the disease progression. The mutation type cannot be related to the different subtypes since a report of a family with 3 affected children each with a different Farber subtype were presented (Amirhakimi et al., 1976).

Considering that the assay confirming Farber diagnosis are not straightforward when percent conversion is very low (under ~4%) and our assay detecting Cer/Chol domains clearly showed distinction only in subtypes 4 and 7 the very severe form of the disease, we conclude that our antibody labeling assay can be potentially used as a diagnostic tool for these severe cases. Our experimental procedure is simple fast and relatively low in costs.

Using both fluorescence and electron microscopy we were able to detect a big elevation in Cer/Chol domains in fibroblast of the two most severe Farber subtypes, type 4 and 7. These two subtypes are the earliest in the onset, both neonatal (Scriver, 1995) and in disease progression /death, and both yield the lowest values in the percent conversion in the ACDase assay. These cells could not overcome the enormous loads of Cer entering their endocytic pathway: this probably contributed to the extreme elevation in the intensity of the Cer/Chol domains.

The existence of the Cer/Chol domains in the two particular subtypes might have a connection to the very different symptoms these patients present relative to the more

classical symptoms present in all other subtypes. Since the literature dealing with Farber disease and even more with the subtypes 4 and 7 is very limited we cannot attribute the Cer/Chol domain elevation to a specific pathway but we believe this finding will provide a new relevant direction to explore the disease mechanism and even the use of existing techniques used for cholesterol extraction as possible treatment for Farber patient.

Conclusion

Conclusion

LDSs are complex diseases caused by a wide range of gene leading to protein defects and characterized by intracellular sequestration of multiple substrates, including GSLs, phospholipids and Chol. The storage-accumulated material can be diverse, and sometimes with no direct relation to the primary metabolic defect and are therefore considered as secondary, however, with a key role in the disease pathogenesis. In normal conditions, membrane components that are delivered to late endosomes and lysosomes are subject to hydrolysis by the hydrolytic enzymes in these organelles. This catabolic process is important for the normal turnover of lipid components, and a lack of activity from one of these hydrolases leads to an accumulation of the undegraded substrate for the missing hydrolase. In most of LDSs, the lipids accumulation can be seen in many tissues, however it can be observed in cultured fibroblast lines from the affected individuals. Usually, the brain is the most affected tissue, leading eventually to neuronal death and neurological complications that are often severe and frequently fatal. For example cholesterol accumulation occurs along with GSLs in many lysosomal diseases and evidence suggests that it may interfere with normal endosomal/lysosomal processing as well as lead to cholesterol deficits elsewhere in neurons, with consequences for neuronal function. Thus it becomes important to understand the cellular and subcellular locations, and the reasons for sequestration of the many secondary storage compounds in order to characterizing lysosomal disease for the full elucidation of the cell biology of these conditions. In the present study, we showed:

1) Perturbations in GM2- Ganglioside and cholesterol homeostasis in FD fibroblasts secondary to SL accumulation.
2) Lipid trafficking alterations by using a fluorescent Cer analogue (BODIPY-C5-Cer)
3) Lysosomal buildup of LacCer by using BODIPY-LacCer, a fluorescent analogue.
4) Elevated levels of cholesterol: C16-ceramide domains in fibroblasts from type 4 and 7 FD patients, the most severe forms.

We speculate that endosomal traffic jams caused by raft alterations in the degradative compartments might contribute to the clinical features associated with each lysosomal storage disorder. Altered trafficking and mistargeting of proteins can also contribute to the complex spectrum of these pathologies.

At the cellular level, further studies to define the intracellular compartment(s) and the mechanisms by which altered SL sorting occurs in FD cells vs normal cells are required. Mechanisms for the redistribution of intracellular cholesterol in FD cells need to be evaluated. Further experiments devised to rigorously test microdomains and intralumenal

membrane-based sorting models for SL targeting should be performed. It will also be important to learn whether the current observations with fluorescent SL analogs extend to endogenous lipids, as suggested by preliminary studies. More work will be necessary to establish how cholesterol and sphingolipids are distributed in cells and how the regulation of their location affects cellular function. In addition to profiling the lipids which accumulate in FD cells, further studies on the linkage between SL accumulation and cholesterol redistribution should be performed by increasing the levels of natural SLs in normal fibroblasts and learning whether intracellular cholesterol redistributes in response to elevated SLs.

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